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INDEX NUMBER

JUL 26 1961

The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 57 JUNE, 1961 NO. 6

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The Official Publication of the Central Society for Clinical Research

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Vol. 57, No. 6, June, 1961. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription rates: United States, its Possessions, and Canada $13.00; Latin America and Spain $14.00; Other Countries $15.00. Students, interns, and resident physicians: United States, its Possessions, and Canada $7.80; Latin America and Spain $8.80; Other Countries $9.80. Single copies $2.00 postpaid. Second class postage paid at St. Louis, Mo. Printed in the U. S. A.
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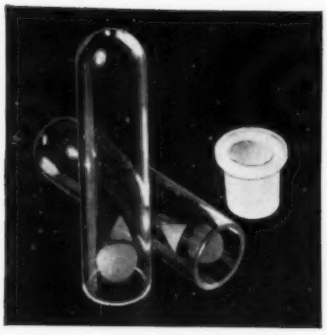

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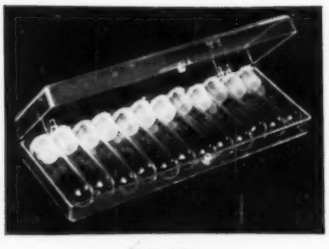

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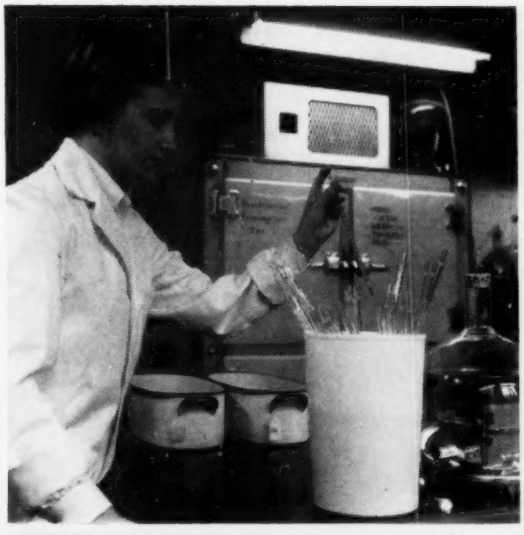

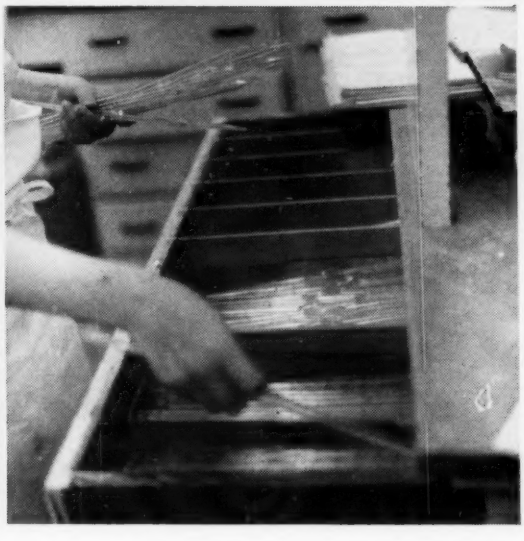

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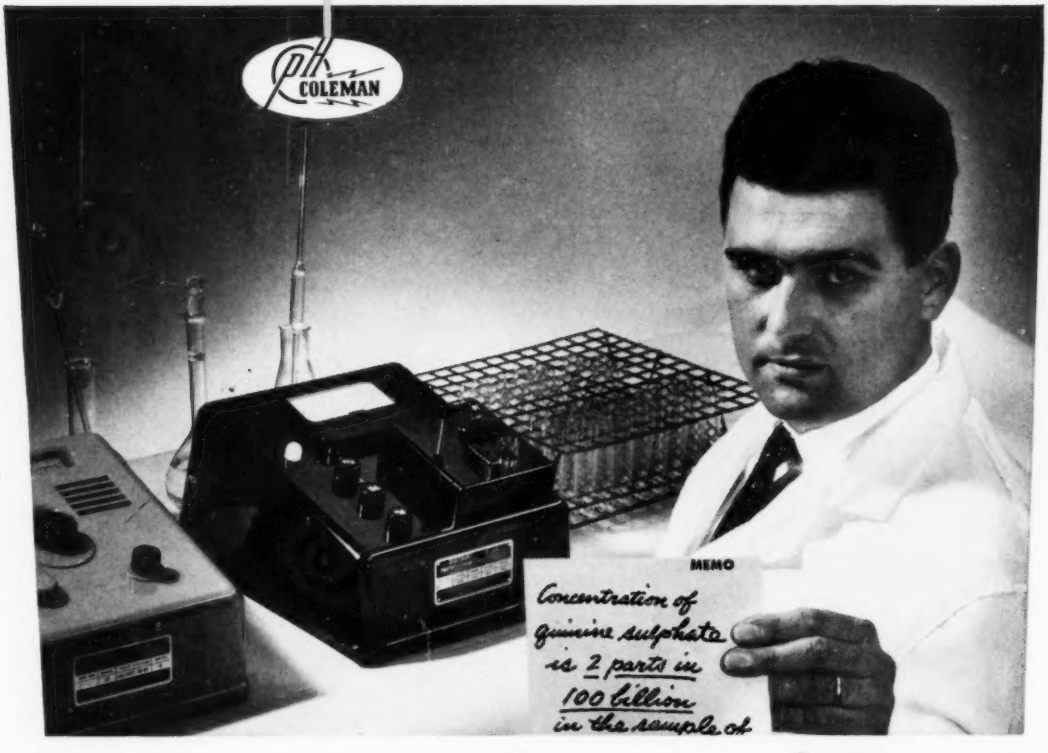

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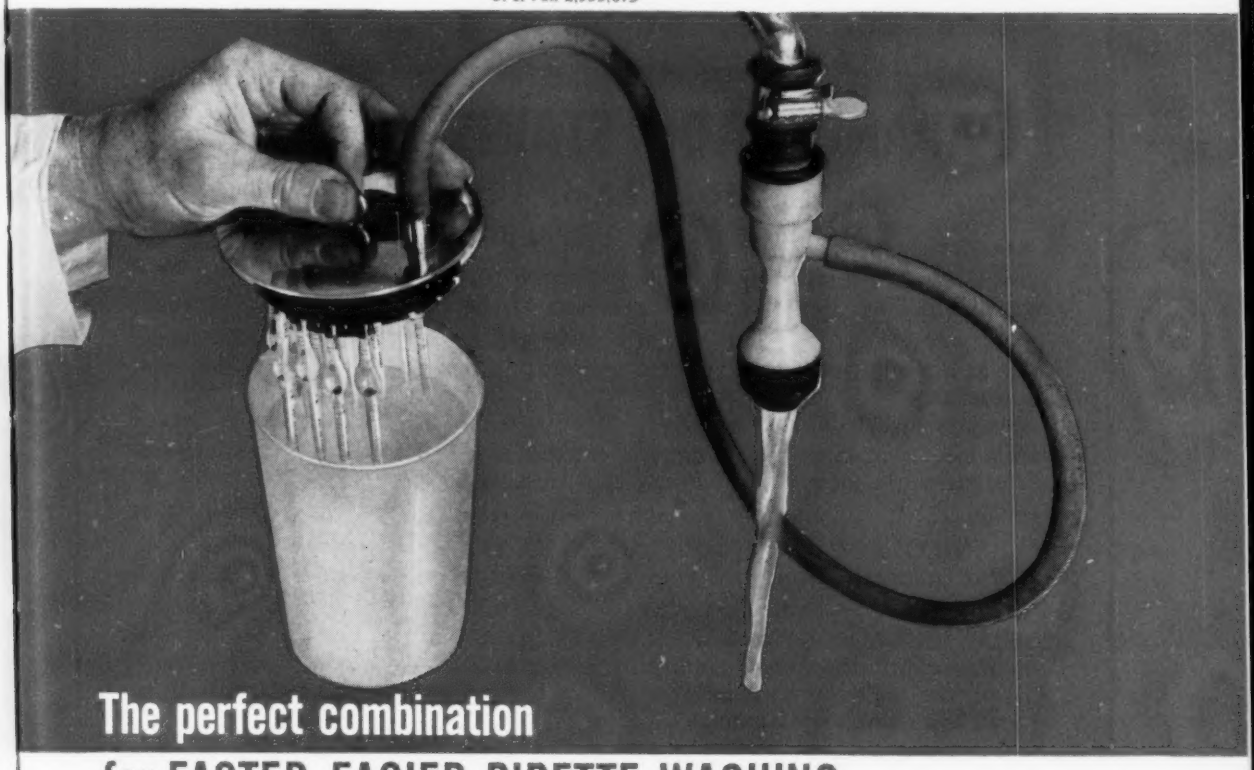

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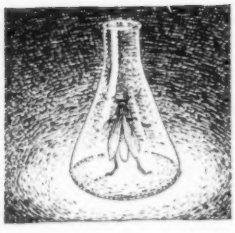
a glance at yesterday in relation to today



IN JUNE—(1301)—a paper read before the Royal Society describes some experiments and observations on "spontaneous light". Herring, mackerel, and other objects "that abound with spontaneous light in the latent state" do not emit such light when deprived of life. Contrary to what other authors have alleged, oxygen does not render this light more vivid than it is in atmospheric air. Azotic gas, on the other hand, is favorable to spontaneous light and helps preserve its emission and brilliancy, while hydrogen, carbonic acid gas, and sulphurated hydrogen gas act to extinguish it.[1]

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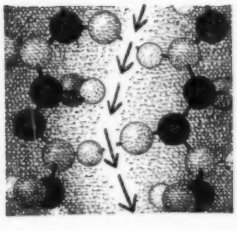

IN JUNE—(1880)—R. H. Chittenden reviews recent work on the enzymatic breakdown of albumin. "... it is plain that by the first step in digestion the albumin molecule is resolved into its two parts [antialbumose and hemialbumose], the gastric juice then readily changing the hemialbumose into hemipeptone, while the anti-group appears less susceptible to the action of pepsin, only a small portion of this group being changed into antipeptone, while the larger part, under the influence of the dilute acid, appears to be converted into partially insoluble products; but on reaching the intestinal canal these insoluble bodies, under the influence of the alkaline solution of trypsin, pass readily into solution and are digested with formation of antipeptone, which is readily absorbed, while the hemipeptone is further changed into crystalline decomposition products."[2]

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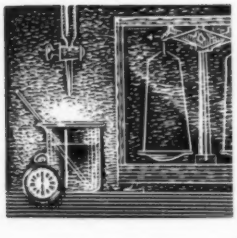

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1. Hulme, N.: A continuation of the experiments and observations on the light which is spontaneously emitted from various bodies; with some experiments and observations in solar light, when imbibed by Canton's Phosphorus. Phil. Tr. Roy. Soc. London *91*:403 (June 18) 1801. 2. Chittenden, R. H.: Report on progress in physiological chemistry, Am. Chem. J. *2*:204 (June) 1880. 3. Dische, Z.; Shettles, L. B.; and Osnos, M.: New specific color reactions of hexoses and spectrophotometric micromethods for their determination. Arch. Biochem. *22*:169 (June) 1949.

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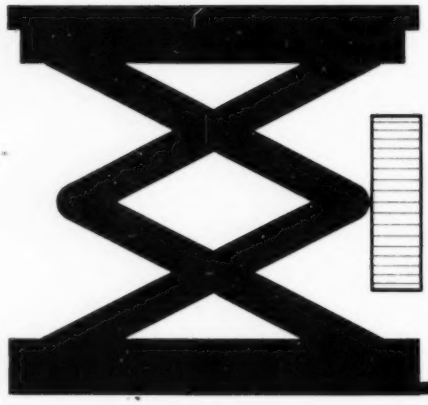

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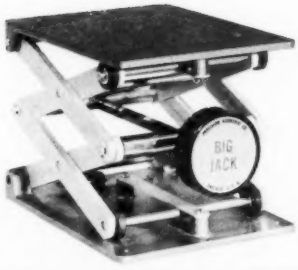

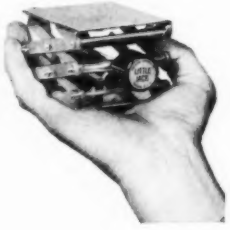

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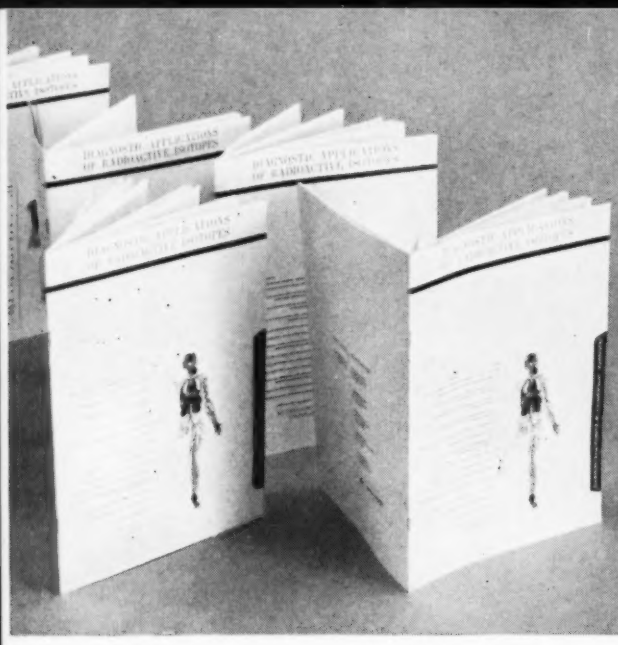

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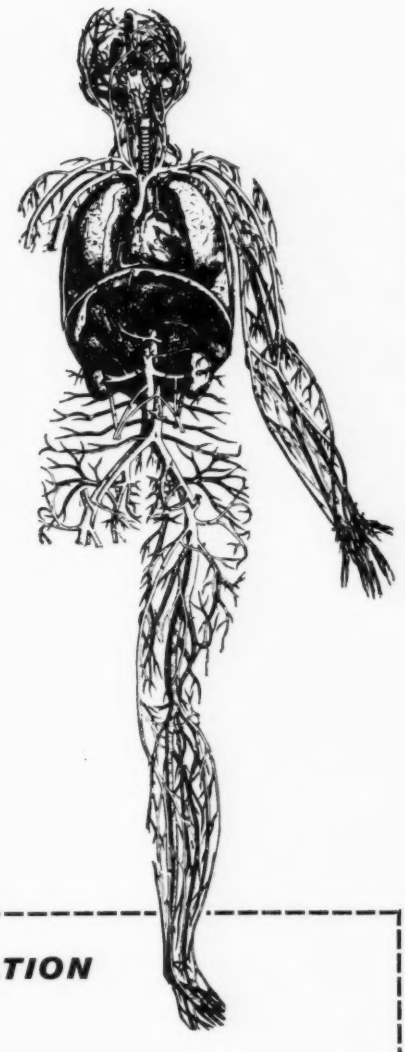

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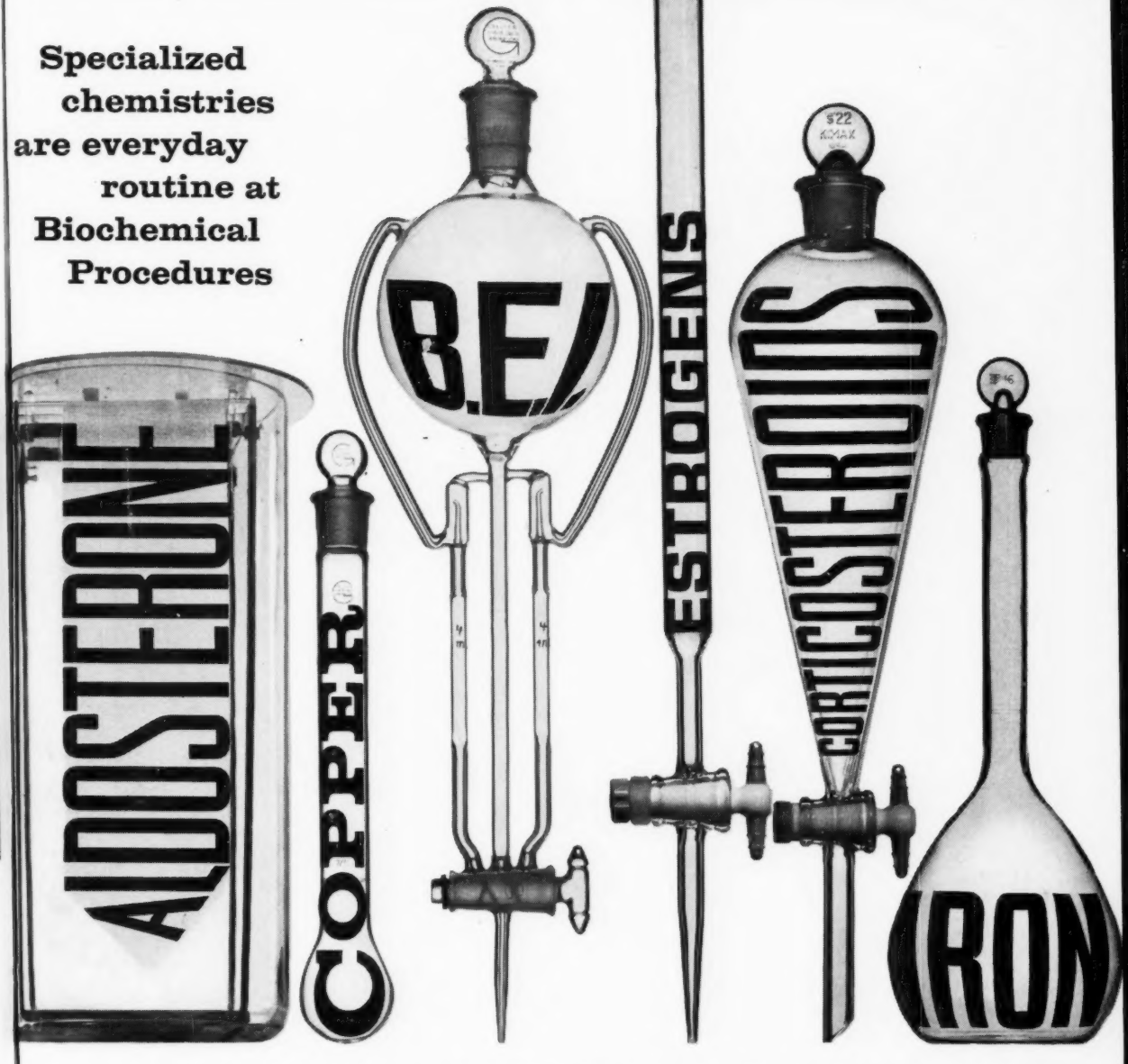

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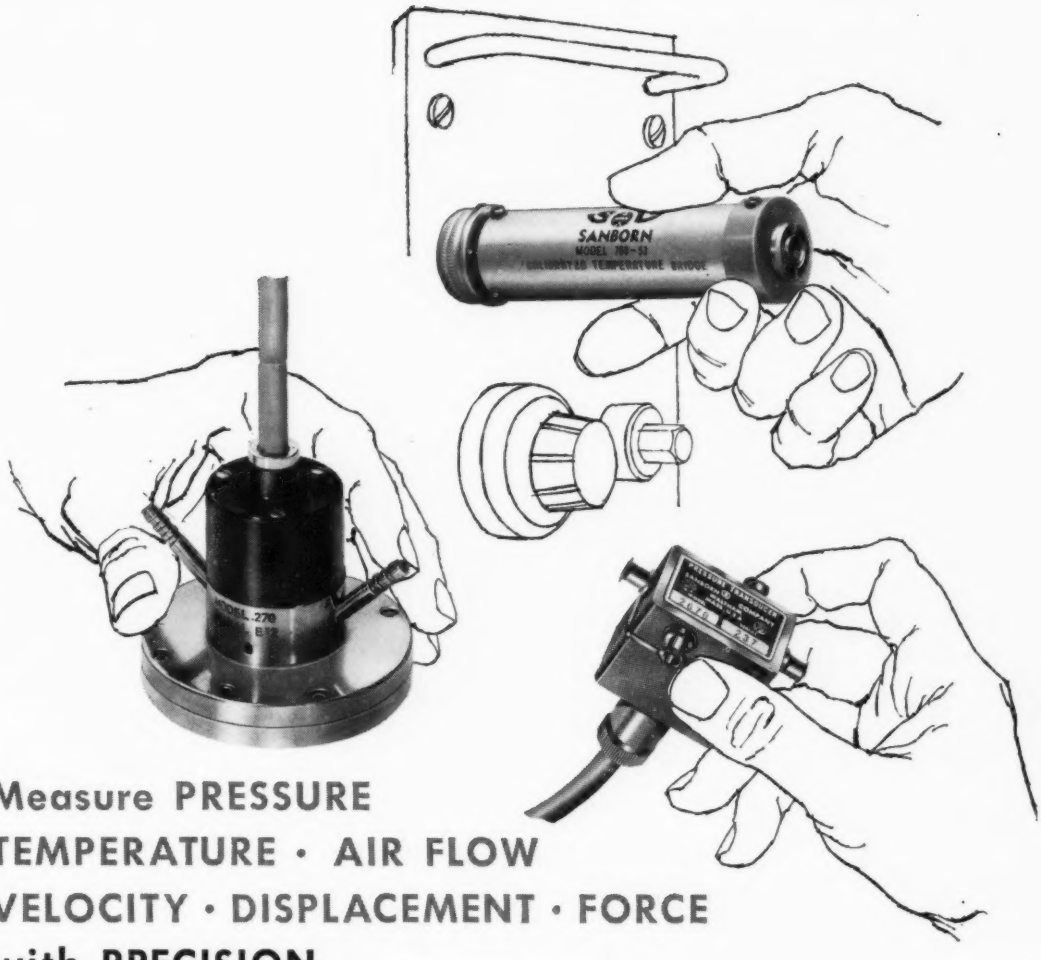

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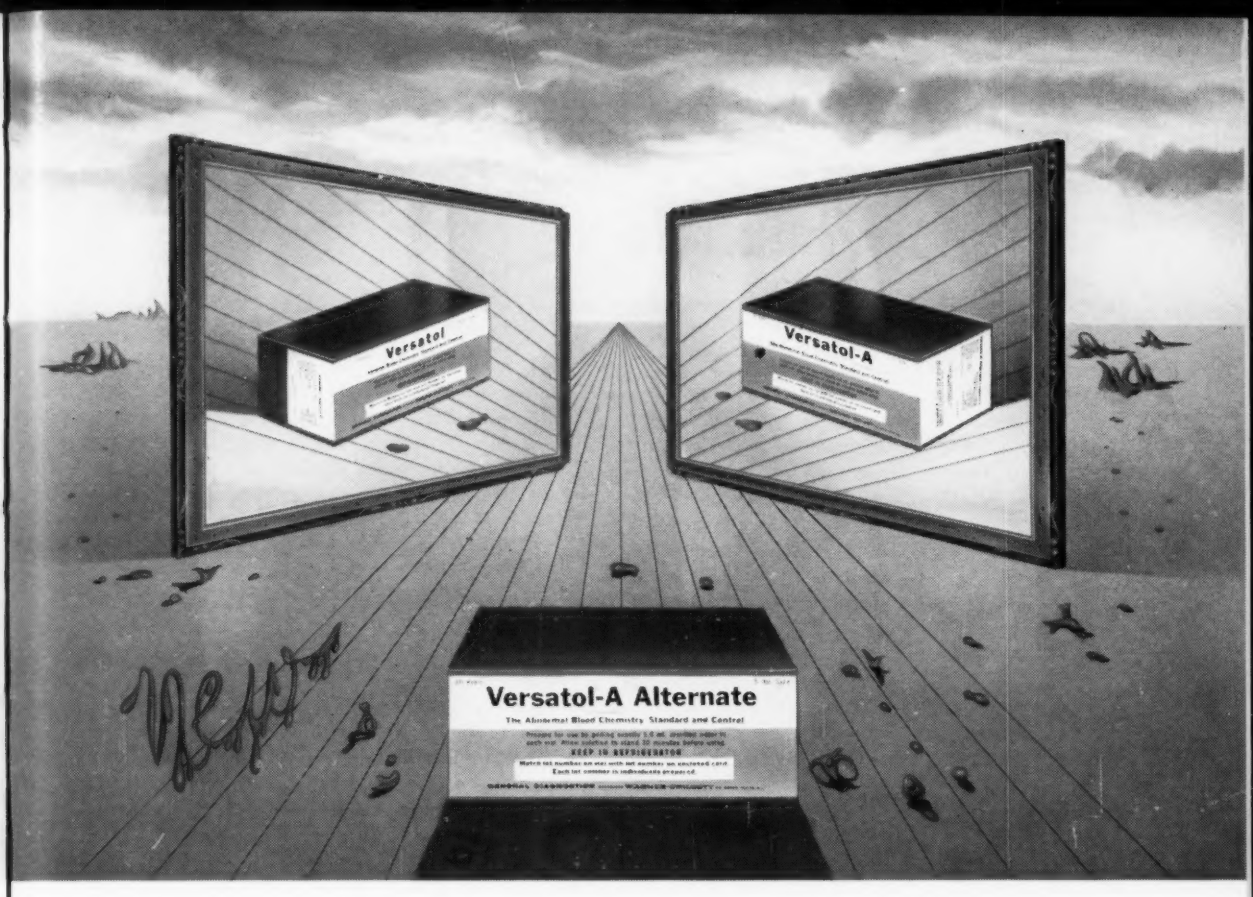

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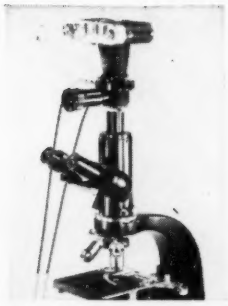

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The Journal of Laboratory and Clinical Medicine

VOL. 57 JUNE, 1961 No. 6

CLINICAL AND EXPERIMENTAL

SERUM HAPTOGLOBINS: THEIR SEMIQUANTITATIVE ESTIMATION BY A PAPER ELECTROPHORETIC TECHNIQUE

EMERY C. HERMAN, JR., M.D.*
BALTIMORE, MD.

This method for the semiquantitative measurement of serum haptoglobin is predicated upon the electrophoretic differences between free hemoglobin and haptoglobin-bound homoglobin. Hemoglobin C, rather than Hemoglobin A, has been used because it affords (at pH 8.6, veronal buffer) a more distinct separation between bound and unbound forms of hemoglobin. The experimental validation for this method is presented. The relative merits of this and other methods for the determination of haptoglobin are discussed. Because of its simplicity, relative rapidity, and use of inexpensive equipment, this method seems suited to the needs of clinical laboratories.

AVAILABLE evidence now clearly indicates that there is an interaction between extracorpuscular hemoglobin and *specific proteins* (alpha$_2$ globulins) found in plasma and serum. Such protein-hemoglobin complexes have recently been found to have an important role in the metabolism of hemoglobin.

Attention was first focused on these specific proteins by Polonovski and Jayle,[1] in 1938, who showed that this complex of hemoglobin and serum possesses enzymatic activities different from those possessed by either component alone. These workers designated as *haptoglobins* the serum proteins which have particular affinity for hemoglobin. By determination of the rates of peroxidation of alkyl peroxides catalyzed by the haptoglobin-hemoglobin complex, they obtained a quantitative measurement of the haptoglobins.

From the Hematology Division, Department of Medicine, The Johns Hopkins University and Hospital, Baltimore, Md.

Supported in part by a research grant from the National Institutes of Health, United States Public Health Service.

Received for publication Oct. 4, 1960.

*Present address: c/o Mary Imogene Bassett Hospital, Cooperstown, N. Y.

More recently, physicochemical means of separation have been used in the study of haptoglobins. Ammonium sulfate and ethanol precipitation techniques have been used to separate Hp* from other serum proteins.[2] Hemoglobin bound to haptoglobin has a mobility different from that of free (i.e., unbound) Hb* as measured by zone electrophoresis on several media.[3-8] The bound and unbound forms of hemoglobin are also separable by column chromatography.[9, 10] The Hp-Hb* complex behaves differently from Hb in an ultracentrifugal field.[11]

In addition to the above enzymatic and physicochemical means of detecting the Hp-Hb complex, evidence has accumulated that the human renal glomerulus handles bound and unbound hemoglobin differently. The importance of this means of "physiological" discrimination has been emphasized in several recent reports,[4, 5, 12] which discuss the bearing of haptoglobins on the pathogenesis of hemoglobinuria. In addition to their importance in various hemoglobinurias, haptoglobins are under scrutiny regarding their role in several types of hemolytic anemias.[6, 8, 13]

In the medical clinics of The Johns Hopkins Hospital many patients with hereditary hemoglobinopathies are encountered, and an investigation of serum haptoglobins in these persons has been undertaken. A simple method has been devised to measure the haptoglobin activity (content) of serum; this method is based upon the observation that bound and unbound hemoglobin display different electrophoretic velocities. The purpose of this paper is to describe this method and its experimental validation. The results obtained in a survey of 150 persons will be reported in another paper.

MATERIALS AND METHODS

Preparation of Serum.—Venous blood was carefully drawn into a siliconed syringe, transferred to siliconed centrifuge tubes, and promptly spun for 3 minutes at 0° C., 14,000 g. To the supernatant native plasma thus obtained ground glass fragments were added to promote clotting. After 2 hours' incubation at 38° C., the glass and fibrin clot were removed. Serum was stored at 4° C. until use. Additional visible clotting occurred at times, and the serum was cleared of fibrin by centrifugation.

Preparation of Hemoglobin Solutions.—Persons known beforehand to have specified hereditary types of hemoglobin (as tested by paper and agar electrophoresis and confirmed by family studies) were used as sources of hemoglobin. According to the method of Drabkin,[14] hemoglobin solutions were prepared from saline-washed whole blood. These solutions were dialyzed against 0.005 M potassium phosphate, pH 7.0, at 4° C., and then were stored at −20° C. at concentrations of approximately 10 Gm. per cent. Aqueous dilutions of this stock hemoglobin were made at intervals of 2 weeks.

Conditions of Electrophoresis.—The electrophoretic separations of sera were accomplished at pH 8.6, 0.05 M sodium veronal, in a horizontal glass plate apparatus previously described for the separation of hemoglobins.[15]

Staining Solutions.—One gram of benzidine base was dissolved in 90 ml. glacial acetic acid, diluted to 100 ml., and stored at 4° C. A 1.5 per cent solution of H_2O_2 was freshly prepared by dilution of 30 per cent H_2O_2 with ice-cold distilled water. Bromphenol blue was dissolved in 5 per cent acetic acid to a final concentration of 0.1 per cent before use.

Measurement of Hemoglobin Concentration.—This measurement in aqueous solution was based on its spectrophotometric absorption at 544 mμ with reference to a standard hemoglobin

*In this report, the following abbreviations will be used: Hp for unbound haptoglobin; Hb for unbound hemoglobin; and Hp-Hb for the complex of haptoglobin with hemoglobin.

concentration derived from measurement of the absorption of cyanmethemoglobin.[16] The molar extinction coefficient for hemoglobins A, S, and C was assumed to be the same. Hemoglobinemia was estimated according to the technique of Crosby and Furth.[17]

Units of Measurement of Haptoglobins.—In this report the measurement of haptoglobin is expressed in terms of the largest amount of hemoglobin bound by 100 ml. serum, i.e., in milligram per cent.

EXPERIMENTAL STUDIES

The Typical Experimental Assay for Haptoglobin Concentration.—This was performed in the following manner: the test serum was pipetted into several small (Kahn) test tubes in volumes previously calculated so that the addition of a known volume of stock hemoglobin would result in the expected final concentration of Hb in serum. This volume of hemoglobin, usually 0.003 ml., was then added to each tube, and was easily dispersed throughout the serum by gentle flicking of the test tube. In tubes in which a very low concentration of hemoglobin in serum was desired (i.e., less than 10 mg. per cent), the mixture was thoroughly mixed by alternately sucking up and expelling the mixture with a capillary pipette. The serum-hemoglobin mixtures, as well as control Hb and serum, were then incubated 30 minutes in a water bath at 38° C.

While the incubation was under way, a 14 by 30 cm. piece of Whatman No. 3 filter paper, which had previously been lined with pencil into parallel longitudinal lines 1 cm. apart, with one centrally placed perpendicular line, was immersed in the buffer and allowed to drain 1 minute. With a micropipette, 0.002 ml. of the serum mixtures was applied in a line of small drops to the midline of the damp paper. In this maneuver it was important to apply the tip of the micropipette to the paper and to expel the fluid gently lest the small drop splatter. In each experiment a control of free Hb and of unadulterated serum was also run, and in those instances where the migration of the patient's own hemoglobin was important it was included.

The paper was then placed between glass plates in the horizontal tank, and electrophoresis was carried out at room temperature for 2½ to 3½ hours at 300 v. potential difference measured at the tank electrode terminals. Under these conditions a current of 10 Ma. was observed. A separation of serum components of 8 to 12 cm. was accomplished. After electrophoresis, the paper was dried in a hot air oven for 10 minutes and was then cut longitudinally into those strips to be stained for protein and those for hemoglobin. The "protein strips" were immersed in bromphenol blue for 1 hour, rinsed with 5 per cent acetic acid and water successively, and dried by hanging in air. The dry strips to be stained for heme pigments were suspended from the window of a ventilation hood.* Approximately 1.5 ml. of benzidine solution and 3 ml. of H_2O_2 were mixed in a spray atomizer† attached to an air jet. The paper strips were thoroughly sprayed. Heme-reactive areas on the paper became a sky-blue color in about 30 seconds; because this color persisted for only 3 to 5 minutes those areas which had been specifically stained were marked promptly by pencil. Comparisons between 2 sera or between the same serum at different hemoglobin concentrations were made immediately after the pencil outlining. Satisfactory photographs of this staining of heme-reactive spots could not be obtained because of the fleeting nature of the blue color.

The sort of pattern obtained is shown in Fig. 1, which illustrates schematically the spread of the serum protein fractions from the gamma globulins on the cathodal side of the line of origin to the albumin which has moved farthest toward the anode. Normal adult hemoglobin (Hb A) moved as shown to approximately the position of the beta globulins, whereas Hb C remained at or near the site of application. If either of these hemoglobins was combined with serum containing Hp, the heme-reactive spot moved to a zone usually between beta and alpha$_2$ globulins. An unequivocal distinction between free and bound

*Acetic acid fumes are quite pungent. Benzidine is considered to be toxic and possibly carcinogenic.

†A deVilbiss No. 40 atomizer served well.

forms of hemoglobin could be made easily in the case of Hb C, but not with Hb A. Unless otherwise mentioned, Hb C, obtained from persons with homozygous Hb C disease, has been used in the studies to be reported.

Determination of Haptoglobin Content.—Hemoglobin is bound preferentially to the haptoglobin(s) of serum, and the quantitative estimation of the Hp of a serum is, by general definition, expressed in terms of the largest amount of Hb which can be bound.[4] In these studies, as long as the amount of Hb added was less than the amount with which the Hp could combine, there was but one hemoglobin-reactive area when the electrophoretic strip was stained. On the other hand, whenever Hb had been added in excess of Hp, there were heme-reactive spots in the Hp-Hb area and in the position expected for free Hb, and at times, methemalbumin also appeared. By gradually increasing the concentration of Hb in the serum, one could, as it were, titrate the hemoglobin binding capacity of the serum. From practical considerations, in the experiments to be reported, ranges of 15 per cent were chosen for titration limits.

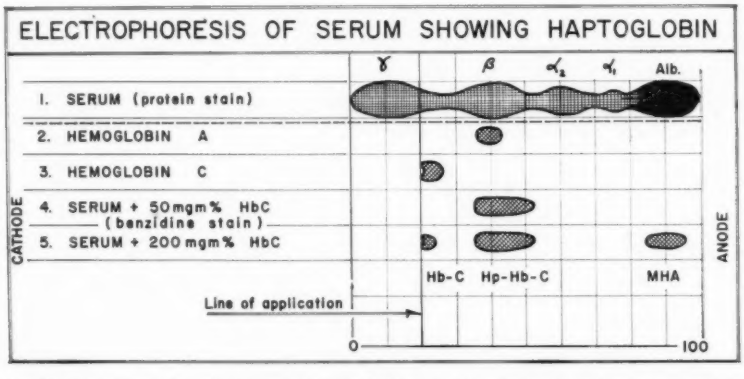

Fig. 1.—Electrophoresis of serum, hemoglobin, and mixtures of serum and hemoglobin. In line 1, a normal serum has been stained with bromphenol blue, and the typical electrophoretic separation of serum components is seen. In lines 2 and 3, hemoglobins *A* and *C* (unstained) show distinctly different migrations. In lines 4 and 5, both stained with benzidine, the heme-reactive area is present only in the haptoglobin area at a concentration of 50 mg. per cent, whereas the heme reaction is present at 3 spots (haptoglobin, free hemoglobin, and methemalbumin) in the concentration of 200 mg. per cent.

RESULTS

Sensitivity for Detection of Hemoglobin.—Normal native serum which contained no hemoglobin was used to make dilutions of 8, 4, 2, and 1 mg. per cent Hb C in serum. After electrophoresis of 0.002 ml. of each of these mixtures, the strips were sprayed: an unequivocal heme-reactive area was present at a concentration of 2 mg. per cent. Moreover, whenever in the clinical studies the native serum contained more than 2 mg. per cent hemoglobin, this amount was detected. The sensitivity was dependent among other things upon the buffer and pH at which electrophoresis was accomplished. When 0.05 M potassium phosphate was used at pH 7.0, the sensitivity was equivocal below concentrations of 10 mg. per cent.

Reproducibility of Haptoglobin Measurement.—Several sera of differing Hp contents were titrated at 15 per cent increments. Duplicate analyses never revealed a deviation of more than one tube.

Is Haptoglobin Activity a Linear Function of Haptoglobin Concentration?—Because of the inexactitude of titration measurements, this question could not

be answered by this technique. However, the following experiment, demonstrated by a graph in Fig. 2, indicates the likelihood that there was a linear relationship between Hp content and Hp activity (i.e., activity with regard to Hb binding). A normal serum was diluted with 1 part and 3 parts water to effect 1:2 and 1:4 dilutions and was compared to the undiluted specimen: the diminution in haptoglobin activity was linearly related to the number of molecules of Hp present. In order to be sure that this did not depend on the dilution of other serum proteins, another experiment was performed in which a serum which contained no Hp (a serum from a person with sickle-cell anemia) was used instead of water as the diluent: similar results were obtained.

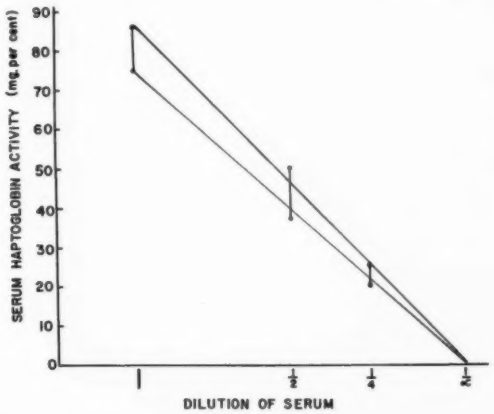

Fig. 2.—The relation of haptoglobin activity to haptoglobin concentration. The ordinate refers to the hemoglobin-binding capacity of the serum and is plotted as the range obtained by titrations. The abscissa is the reciprocal of dilution and is plotted according to the theoretical number of molecules of haptoglobin present.

Is the Affinity of Haptoglobin for Hb C the Same as for Hb A?—Because of the decidedly greater reliability in discrimination between Hp-Hb C and Hb C than between Hp-Hb A and Hb A, the above question is of paramount importance. A normal serum from a person with Hb A was titrated independently with hemoglobins A, S, and C in parallel experiments. Under the conditions of this experiment the complete separation of free and bound Hb A was not possible. Nevertheless, free Hb S and Hb C appeared at the same titration limits, and this was the same concentration at which free Hb A seemed to appear. Serum from a person with homozygous Hb C disorder was titrated similarly. The Hp content was the same when either Hb A or Hb C was used.

Normal serum was next titrated by the use of 50:50 mixtures of hemoglobins C with A and C with S. In such an experiment, if the affinity of Hp for one of the hemoglobins were greater than for another, a difference would be shown between the titration limits when A-C, C-S, and C-C were used. No difference was seen; hence the affinity for each of these hemoglobins was the same. Not only did various human hemoglobins behave similarly by this technique, but the haptoglobin content of dog serum was shown to be the same whether measured with Hb C or with dog Hb.

There were, however, some differences between the complex of Hp-Hb A and of Hp-Hb C of the same serum. The mobility of the Hp-Hb complex with Hb A was slightly more rapid than that of the complex with Hb C. On paper electrophoresis this difference was slight. It could be accentuated by electrophoresis in starch gels,* and differences in sedimentation velocities between the two types of complexes have been reported.[18] The fact that these qualitative differences exist, however, does not mean that the quantitative affinity for the various hemoglobins is not the same.

Effect of Storage.—From a normal person native plasma was obtained in the usual manner. A portion was clotted and the serum expressed; other portions were stored as the unclotted plasma at –20° or +4° C. for a period of 3 weeks. The serum was stored at –20°, +4°, and +25° C. for a 3 week period. One tube was frozen and thawed 5 times during the storage period. At the end of the 3 weeks' storage each of these specimens had a Hp content which was 70 to 100 per cent of the original.

Effect of Incubation Time.—The interaction of Hp with Hb was rapid, and no differences between incubation times of 5, 30, and 120 minutes could be demonstrated.

Some Characteristics of Haptoglobin Binding.—The affinity of Hp for Hb was such that, once the complex was formed, the Hb could not be displaced in vitro by the addition of more Hb. The Hp of a serum was partially saturated with Hb C. After incubation, Hb A (in excess of the Hp content) was added; this mixture was reincubated. After electrophoresis, free Hb A but not free Hb C was present. On the other hand, when simultaneous addition of the above same amounts of Hb C and Hb A were made to the serum, subsequent electrophoresis showed both free Hb A and free Hb C in addition to the Hp-Hb.

Haptoglobin combined in essentially the same way, both quantitatively and qualitatively, with methemoglobin as with oxyhemoglobin. If a serum contained methemalbumin, however, the addition of exogenous haptoglobin did not displace the heme moiety from the albumin.

Sources of Error in Quantification of Haptoglobin.—Because this method depended upon the accurate delivery of a micro-amount of concentrated hemoglobin to a relatively small volume of serum (the volumes of serum were not less than 0.15 ml.), it was subject to errors of pipetting. Meticulous attention to the matter of clean pipettes was necessary, and the same pipette was used in making each dilution for a given experimental series.

If the native serum were incompletely clotted before addition of hemoglobin, the formation of a small amount of fibrin which remained at the point of application might trap some Hp-Hb and create the illusion of free Hb C having been present. Such a fibrin trap did not result in a symmetrical teardrop pattern of hemoglobin-reactive material; rather, it was an asymmetric tiny dot. The difference was usually easily noticed. Such a discrepancy could be eliminated by ensuring complete clotting beforehand.

*Dr. Carmen Merriman, The Johns Hopkins Hospital, Department of Medicine, performed these starch gel electrophoreses.

DISCUSSION

Of the several methods used to determine haptoglobins, the measurement of peroxidase activity[1, 10, 19] is the oldest. This method affords a precise measurement rather than a range, but it has several drawbacks in clinical practice. It fails to demonstrate whether there is Hp-Hb complex present in the native serum, since this serum is arbitrarily assumed to have zero Hp-Hb catalytic properties. It would, thus, not indicate accurately basal haptoglobinhemoglobinemia, in which situation free Hp might not be present.

Haptoglobin has not yet been isolated as a pure protein so that specific analysis of free Hp is not possible. Inasmuch as it accounts for a variable proportion of the alpha$_2$ globulins, one cannot use measurements of these proteins as a satisfactory estimate of serum Hp.[20] Smithies and Walker[3] have used the technique of starch gel electrophoresis to delineate the hereditary types of serum Hp, but for the purpose of measuring serum Hp[4] these techniques are cumbersome in that a long period of electrophoresis is required and the number of determinations per starch gel is few. Likewise, the sedimentation of Hp by ultracentrifuge[13] represents too formidable an undertaking for the clinical laboratory. The use[9, 10] of ion-exchange resins in the column chromatography of Hp affords not only a chance for accurate quantification of Hp but also the opportunity to obtain these proteins in significant quantities. With the increasingly frequent use of this powerful resolution technique, many new approaches to the metabolism of Hp and of Hb become possible.

Most clinical laboratories perform paper electrophoretic analysis of hemoglobins and serum proteins. Electrophoresis between glass plates as used in this study is readily adaptable to Hp measurement also, and the apparatus is inexpensive and sturdy, requiring minimal upkeep repair. Because the pH of 8.6 chosen for the measurement of haptoglobins in this study is the same as that commonly chosen for the electrophoretic separation of various hemoglobins and of serum proteins, a single electrophoretic run can serve for the study of each of these protein components. The use of Hb C in these studies is of value solely in that the end point can be recognized more accurately. The rarity of persons with hemoglobin C disorder is no real handicap in that persons with Hb C trait (i.e., A-C) would serve equally well. The use of Hb S allows easier recognition of end points than does Hb A, but is not as good in this regard as is Hb C.

Other workers have used the hanging-strip paper electrophoretic apparatus with good results. Neale and associates[6] have used this technique with normal adult hemoglobin; at pH 8.6, the customary pH for the screening of human hemoglobins on paper, the separation between Hp-Hb A and Hb A is not distinct in this apparatus. Laurell and Nyman[5] and Lathem and Worley[7] have used phosphate buffer at pH 7.0 and thus have achieved a much better separation of Hp-Hb A and Hb A. The practice[7] of adding a considerable excess of hemoglobin (200 mg. per cent concentration) to serum and then of analyzing the relative densities of staining of the Hp-Hb and free Hb areas gives one a precise measure obtained from one experimental dilution instead

of the range afforded by a titration series. Implicit in the accuracy of this measurement are the assumptions that equal amounts of heme, either as Hp-Hb, Hb, or as methemalbumin yield similar benzidine color, and that elution of color during staining occurs at the same rate from all 3 pigments: both these assumptions have been confirmed experimentally. This method would seem admirably suited for any laboratory which has the needed equipment.

By definition, in these studies the measurement of Hp has been referred to the affinity of a serum for *additional* hemoglobin. By virtue of the care used in obtaining the native sera we have not encountered adventitious hemolysis greater than 4 mg. per cent in normal persons, thus this small amount is not of concern. However, in certain persons with hemolytic anemia in whom hemoglobinemia is regularly seen,[21] the amount of hemoglobin in native serum may be considerable, so that the reference point for Hp measurement should be clearly stated.

SUMMARY

A method for the semiquantitative estimation of serum haptoglobins is presented. This method is based on the different electrophoretic mobilities of bound and unbound hemoglobin, and differs from others heretofore used in that hemoglobin C is used instead of hemoglobin A. The method requires equipment and techniques easily adapted to routine clinical laboratories. The experimental validation of the method is presented.

I wish to thank Anne Bjerklie for her assistance in all the technical phases of this work, and Drs. E. D. Thomas and W. B. Greenough and Mr. O. Clute for helpful criticisms of the manuscript.

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SERUM HAPTOGLOBINS IN HEMOLYTIC DISORDERS

EMERY C. HERMAN, JR., M.D.*
BALTIMORE, MD.

Serum hemoglobin and haptoglobin values were determined in a group of normal persons and in persons with hematologic disorders which included hemolytic anemias. In normal persons, serum hemoglobin was never greater than 4 mg. per cent, and the serum always contained free haptoglobin, usually in excess of 50 mg. per cent. In persons with hemolytic anemias, free haptoglobin was generally absent from serum, although a haptoglobin-hemoglobin complex was frequently found. The serum hemoglobin was often elevated in several sorts of hemolytic disorders, notably sickle-cell and pernicious anemia, but was not elevated in hereditary spherocytosis. Evidence was obtained to indicate that the absence of haptoglobin in hemolytic anemia is a result of depletion of haptoglobin and not of a failure of synthesis. The absence of haptoglobin from the serum nearly always signified hemolytic disorder, but haptoglobin was found in sera from several patients with presumably hemolytic anemia. The factors bearing upon the metabolism of haptoglobin in these persons are discussed.

NEITHER in normal persons nor in persons with hemolytic disorders have the processes of destruction of the erythrocyte and its constituents been fully elucidated. One of the first considerations is the locus of liberation of hemoglobin and its subsequent fate: under what circumstances does this liberation happen in the intravascular compartment, and when does it occur within the reticuloendothelial system?

There are specific serum globulins, the *haptoglobins* (Hp), which can bind extracorpuscular hemoglobin (Hb) both in vitro and in vivo. Investigators[1-6] of the physiologic role of Hp have recently shown that the serum of persons with active hemolytic anemias does not contain Hp, and such findings have led to the hypotheses: (1) that haptoglobins are not synthesized properly; or (2) that haptoglobins are absent because of continuous depletion. Implicit in the latter (and generally favored) assumption is that there is at some time during hemolysis a release of free Hb into the vascular system. A study of the presence of free Hb and of haptoglobin-bound hemoglobin (Hp-Hb) in the serum of persons with hemolytic anemias might indicate where Hb is released and how it is handled subsequently.

From the Hematology Division, Department of Medicine, The Johns Hopkins University and Hospital, Baltimore, Md.

Supported in part by a research grant from the National Institutes of Health, United States Public Health Service.

Received for publication Oct. 4, 1960.

*Present address: c/o Mary Imogene Bassett Hospital, Cooperstown, N. Y.

Persons with hereditary hemoglobinopathies, outspoken hemolytic anemias, miscellaneous hematologic and other disorders, and a group of healthy normal control subjects have been surveyed for the presence of hemoglobinemia and serum haptoglobins. The results form the basis for this report.

MATERIALS AND METHODS

Hemoglobin, in serum or urine, was measured according to the method specified by Crosby and Furth[7] for plasma and urine. In those cases with very little hemoglobinemia the recommended volumetric changes designed to augment the accuracy of the determination were used. The term "hemoglobinemia," as measured by this method, and as referred to later in the clinical studies, means the *total heme-pigment concentration* of the serum. Because of general usage, the less precise, but more customary, term "hemoglobinemia" will be used.

Measurement of Hp was performed according to an electrophoretic method previously described.[8] Haptoglobin per se was not measured directly, but rather as the capacity of a serum to bind additional Hb: the greatest amount of added Hb which was bound by the Hp of a serum was the "haptoglobin level" (in milligrams per cent) of that serum. This technique[8] was discontinuously titrimetric, thus affording a range rather than an exact measure of Hp. The lowest titer used was 5 mg. per cent, which was well within the limits of sensitivity of detection. If the Hp content was greater than 100 mg. per cent it was rarely measured further. Values between 5 and 100 mg. per cent were measured at titer increments of ½ of the preceding concentration titer.

Collection and Preparation of Serum Samples.—Blood was obtained by means of careful venipuncture using siliconed syringes without anticoagulants. Serum was prepared and stored as previously described.[8] Only those samples obtained and prepared as above were tabulated (Table I) with respect to hemoglobinemia. Serum Hb values (varying from 0 to 15 mg. per cent) from samples collected otherwise were not tabulated, although such sera were included in the serum Hp tabulations.

RESULTS

A. Normal Individuals.—A group of 26 healthy persons, both male and female, Negro and Caucasian, were studied. Twelve pregnant women, none with hematologic disorders, were also included in this category.

Results of studies of serum Hb and Hp are presented in Table I. The level of hemoglobinemia was considerably less than 4 mg. per cent in each case. Moreover, 10 of these sera contained no detectable hemoglobin at all. The mean level of hemoglobinemia was 0.8 mg. per cent and the median was 0.3 mg. per cent. In most cases, the trivial amount of hemoglobinemia found in the normal persons was not detected after electrophoresis of the serum. Whenever heme pigment was detected, it was only at the Hp-Hb area, never at the site for free Hb or methemalbumin.

The serum Hp values are also shown in Table I. About 90 per cent had Hp greater than 50 mg. per cent. The values in pregnant women were generally lower than in the control group, but there was detectable Hp in all subjects.

B. Sickle-Cell Anemia.—Persons in this category were derived from both adult and pediatric groups. Each of them showed the following: (1) a paper electrophoretic pattern of Hb S-S; (2) sickling of erythrocytes in sodium metabisulfite; (3) the confirmation of Hb S, and not Hb D, by agar electrophoresis; (4) a clinical course characterized by variable degrees of continuous anemia, jaundice, and recurrent crises; and (5) insofar as studied, there were no incompatible family data.

TABLE I. SUMMARY DATA CONCERNING HEMOGLOBINEMIA AND SERUM HAPTOGLOBINS

TYPE OF SERUM SAMPLE	NO. OF PERSONS STUDIED	SERUM HB (MILLIGRAMS PER CENT)						SERUM HP (MILLIGRAMS PER CENT)				
		< 0.5	< 1.0	< 2.0	2.0-5.0	5.0-20	> 20	< 5	5-20	20-50	50-100	> 100
A. Normal persons	26	13	0	9	1 (3.7) (2.0)	0	0	0	0	3	10	13
Pregnant women	12	5	2	1	3 (2.5) (2.9)	0	0	0	2	4	1	5
B. Sickle-cell anemia	23	1	1	3	4	6	2 (38.0) (42.4)	18	0	1	1	3
C. Hemolytic anemias:	14											
1. Hereditary spherocytosis	6	0	1	1	2 (3.1) (2.6)	0	0	5	0	0	1	0
2. Pernicious anemia	3	0	0	0	0	0	3 (28.4) (50.0) (48.8)	3	0	0	0	0
3. Autoimmune	3	0	1	0	0	0	0	3	0	0	0	0
4. Other	2	0	0	0	1 (6.6)	1 (17)	0	2	0	0	0	0
D. Hereditary hemoglobinopathies:	27											
1. S-A	9	1	2	1	1 (3.2)	0	0	2	0	0	5	2
2. S-C	4	1	2	0	1 (2.1)	0	0	1	0	1	1	1
3. S-other (J, thal, F)	4	1	1	1	0	0	0	1	1	0	0	2
4. C-C	3	0	1	0	1 (2.9)	0	0	1	0	2	0	0
5. Traits (C, D, F, thal, Ho_2)	7	2	1	3	0	0	0	0	0	2	2	3
E. Miscellaneous disorders:	46											
1. Probably normal	9	2	3	1	0	0	0	1	0	2	3	3
2. Leukemia, lymphoma	8	4	1	2	0	0	1 (44.2)	2	0	0	3	3
3. Miscellaneous hematologic	13	3	2	3	(2.0) 2 (2.9)	1 (7.3)	0	1	0	3	4	5
4. Hepatic	7	1	1	2	2 (2.0) (3.4)	0	0	1	0	2	1	3
5. Renal	2	0	1	0	1 (2.4)	0	0	0	0	0	0	2
6. Other	7	2	1	2	0	1 (11)	0	0	0	2	0	5

The marked differences between the findings in this group and in the normal individuals can be seen in Table I. In the sickle-cell anemia population, a level of hemoglobinemia greater than 5 mg. per cent was found in nearly half the patients. Electrophoresis of the native serum of these persons was done to characterize further the nature of this hemoglobinemia. In the case of a few sera with hemoglobinemia less than 1 mg. per cent, heme-pigment areas were not seen after staining. A few individuals exhibited heme-reactive spots just before the beta globulins and at the site corresponding to albumin. The former site corresponded exactly to the migration of free Hb S; the latter spot was that of methemalbumin. In 14 of 23 instances, there was a definite heme-reactive spot in the Hp-Hb area and usually in the above-mentioned areas as well.

In order to ascertain whether the hemoglobinemia found in many sicklemic persons was at least in part accounted for by Hp-Hb, additional studies were performed. Because of the proximity of free Hb S and Hp-Hb S under the usual experimental conditions, the sera of 2 patients were subjected to electrophoresis in 0.05 M potassium phosphate buffer, pH 7.0. Such conditions afforded a distinct separation of free and bound forms of Hb S and clearly indicated the presence in these 2 sera of a small fraction migrating at the position of Hp-Hb S. In another experiment, the native serum hemoglobinemia was 38 mg. per cent; electrophoresis yielded 3 heme-pigments migrating the same as Hb S, Hp-Hb S, and methemalbumin. This serum was mixed in 3:1 proportions with a normal serum (Hp content $> 145 < 175$ mg. per cent), and the mixture was separated electrophoretically. The free Hb S spot disappeared, the Hp-Hb S spot became darker, and the methemalbumin spot did not change noticeably. This indicated a binding of the free Hb S of the native serum to the exogenous Hp. The migration of the presumed endogenous haptoglobin complex was the same as that of the exogenous, known haptoglobin complex. The heme complex attached to albumin was not dissociated by excess exogenous Hp, even after overnight incubation.

Most persons with sickle-cell anemia who manifested hemoglobinemia had at least some of this Hb bound as Hp-Hb. On the other hand, the capacity of the serum of these individuals to bind *additional* Hb was markedly limited, less than 5 mg. per cent in 18 of 23 persons. The size of the free Hb spot which was due to the added Hb indicated that hardly any had been bound, suggesting that the capacity to bind additional Hb was not only less than 5 mg. per cent but probably was zero. In sera which contained no Hp, there was no factor which inhibited binding by exogenous Hp. There was thus in sickle-cell sera a simple absence of free haptoglobins and not the presence of an "antihaptoglobin."

The sera of 5 unrelated individuals showed normal amounts of Hp. Since the finding of unbound haptoglobins has not been reported in sickle-cell anemia, a brief clinical description of these 5 individuals follows.

R. N., a man aged 33 years, had been anemic for many years, with hematocrit values of 28 to 32 per cent, but with no crises during adult life. He did not show the typical

asthenic habitus, leg ulcers, or visible icterus, and, in general, had had a mild disorder in comparison with his sister, who also had Hb S-S. On 3 occasions over one month he had Hp measurements of $> 66 < 100$, $> 80 < 120$, $> 5 < 10$ mg. per cent without significant hematologic or clinical variation. On the above occasions serum Hb was 1.4, 1.1, and 2.0 mg. per cent, respectively.

L. P., a woman aged 23, twice during her pregnancy showed Hp values of $> 100 < 150$ and $> 20 < 30$ mg. per cent with serum Hb values of 0.9 and 1.1 mg. per cent. Her childhood history indicated rather severe disease, but her morbidity during adulthood had been less than usual for such persons.

B. A., a woman aged 27, with asymptomatic anemia and a relatively uncomplicated past history, was thin and asthenic and showed moderate splenomegaly. A family study was incomplete, but all laboratory studies indicated sickle-cell anemia, and she did not show increased Hb-F. Serum Hp was > 100 mg. per cent; serum Hb was 0.0 mg. per cent.

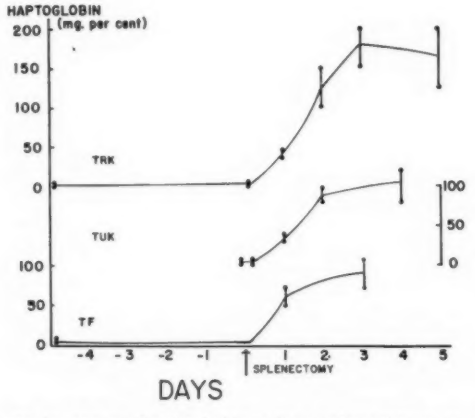

Fig. 1.—The serial changes in serum haptoglobin after splenectomy in persons with hereditary spherocytosis. The ordinates for the 3 persons studied are indicated separately.

J. W., a woman aged 40, except during her "old age" had shown a typically severe course with persistent anemia and jaundice, crises, leg ulcers, and asthenic habitus. Because of the leg ulcers she was admitted to the hospital and given transfusions, with a hematocrit rise from 16 to 40 per cent. One week later, at a time when paper electrophoresis of the Hb showed only transfused Hb A, the serum Hp level was $> 45 < 60$ mg. per cent. One month later, after the hematocrit had fallen to 28 per cent and Hb S had reappeared in the blood, the serum Hp was less than 5 mg. per cent.

B. W., a boy aged 6, had constant anemia, recurrent joint pains, abdominal crises, and attacks of priapism. Haptoglobin was > 100 mg. per cent; serum Hb was 0.5 mg. per cent.

The first 3 of these individuals had had much less difficulty with their disorder than have most persons with sickle-cell anemia. The question of whether Patient J. W. had transfused or native Hp was not settled. Nevertheless, she and Patient B. W. demonstrated the disorder in its customarily severe clinical manifestations. In each of these persons the native serum Hb was considerably lower than the average of those sicklemic persons who possessed no capacity to bind additional Hb.

C. Hemolytic Anemias.—

1. Hereditary spherocytosis: Six persons with this disorder were studied; 3 of these were studied sequentially after splenectomy. None of these persons

had elevated hemoglobinemia levels. Except for one, a 76-year-old woman, all had no capacity to bind additional Hb. The elderly woman, despite having had no transfusions for years, had Hp of $> 50 < 75$ mg. per cent.

The prompt postsplenectomy rise of the serum haptoglobins was most impressive in each of 3 instances; the serial changes are depicted in Fig. 1. In each person unbound Hp was detected before 24 hours after operation.

2. Addisonian pernicious anemia: Although pernicious anemia is not considered primarily a hemolytic disorder, a shortened survival of the megalocytic cells is regularly observed.[9] Three Negro women were studied; each presented profound anemia and megaloblastic erythropoiesis and responded in expected

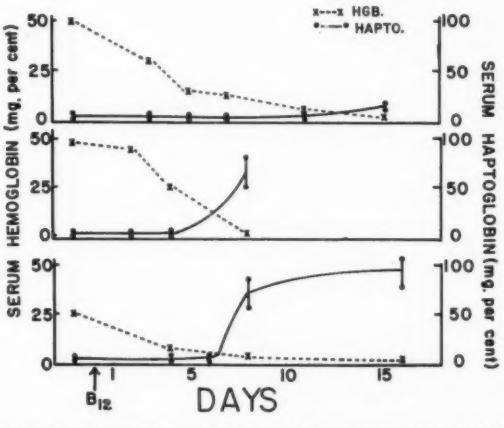

Fig. 2.—The serial changes in serum hemoglobin and in serum haptoglobin following vitamin B_{12} administration to persons with pernicious anemia.

fashion to vitamin B_{12}. Each patient showed marked hemoglobinemia and no Hp prior to therapy. Indeed, the serum was macroscopically tan, rather than pink, most likely the result of interaction of brown methemalbumin with red Hb and yellow bilirubin. Hemoglobinemia persisted for 4 to 11 days following specific vitamin therapy, and not until this had fallen below 4 mg. per cent did Hp appear in the serum. The sequence of events is depicted graphically in Fig. 2.

3, 4. Other hemolytic anemias: Autoimmune hemolytic anemia was encountered in 3 patients, each showing no capacity to bind additional Hb. One woman with paroxysmal nocturnal hemoglobinuria was studied. She showed 6.6 mg. per cent hemoglobinemia and no Hp. The Hb in native serum was possibly bound in part to Hp, but the proximity of free Hb A and Hp-Hb A made this open to doubt, and other substantiating tests were not performed. A 3-month-old boy with an unspecified congenital hemolytic disorder showed 17.4 mg. per cent hemoglobinemia, part of which was bound as Hp-Hb, although he had no capacity to bind any added Hb.

D. Hereditary Hemoglobinopathies.—

1. Sickle trait disorder: No instance of hemoglobinemia above normal values was encountered. Although the serum Hp values were somewhat lower than

were seen in normal subjects, all persons except 2 had Hp. One exception, D. P., a woman aged 29, had had for years mild anemia, reticulocytosis, and jaundice, and had manifested increased anemia during pregnancies. She had no recognizable stigmas of thalassemia minor and no indication of serum hemolysins, Coombs-reactive antibody, or of low levels of glucose-6-phosphate dehydrogenase. She had persistent leg ulcers without known cause. Family studies of her parents were not possible. On 3 occasions during and after pregnancy she had no detectable Hp. Her illness was poorly understood, but 2 of her 6 children, one girl with Hb S-A (the second exception) and one girl with Hb A-A, lacked serum Hp. Her 4 other children, each with Hb A-A, had normal values of serum Hp.

2, 3. Other sickling disorders (Table I): Persons with the hemoglobinopathies of S-C, S-thal, S-J, and S-F[10] showed normal levels of serum Hb, but 2 individuals had no Hp. In the others, Hp content ranged from normal to rather low amounts. The 2 persons without Hp were not noticeably different from those with Hp.

4, 5. Nonsickle abnormal hemoglobins (Table I): In this group were 3 persons with C-C, two with A-thal and A-F,[10] and one person with A-C, A-D, and A-Ho_2.[11] None showed abnormally elevated serum hemoglobin values. One person with C-C hemoglobin had no Hp, but was not clinically different from the other 2 persons who did. As a whole, this group, like the Hb S heterozygotes, had slightly lower serum Hp contents than did the control group.

E. Miscellaneous Disorders.—A group of persons with severe nonhemolytic anemias, and others with several disorders—both hematologic and nonhematologic—were surveyed and have been presented in Table I. In only 3 of 39 determinations was the serum hemoglobin greater than 4 mg. per cent: a young pregnant Negro woman with iron deficiency anemia (7.3 mg. per cent); an elderly moribund woman with a systemic connective tissue disorder (11.0 mg. per cent); and a 60-year-old man with acute monocytic leukemia who had received 2 units of bank blood in the past 48 hours (44.2 mg. per cent).

Five persons showed no free Hp: the 9-year-old daughter of D. P., previously mentioned in the section on sickle trait; a man with acute monocytic leukemia and hemolytic anemia; a young woman with infectious hepatitis; an elderly man 3 hours after transfusion of incompatible blood; and an elderly man with chronic lymphocytic leukemia with mild anemia, but neither reticulocytosis nor a positive Coombs antiglobulin test.

Two individuals with unusually high levels of Hp were encountered: an elderly man with multiple myeloma and a large $alpha_2$ globulin peak ($Hp = > 400 < 600$ mg. per cent), the urinary protein not binding added Hb; and a person with chronic uremia and anemia ($Hp = > 280 < 400$ mg. per cent).

F. Infusions of Hemoglobin.—The binding of Hb by Hp has recently been shown[1, 12] to account for the long-recognized observation that hemoglobinuria does not occur in normal people until serum hemoglobin values exceed 100 mg. per cent, an observation once erroneously attributed to the "renal threshold." In order to observe the mechanisms governing hemoglobinuria in 3 persons with

hemolytic disorders without serum Hp (one with hereditary spherocytosis and 2 with sickle-cell anemia), infusions of autogenous Hb were continuously administered intravenously at rates of 42, 15, and 18 mg. per minute. Bladder urine samples were obtained at 10 to 15 minute intervals by means of an indwelling catheter. In these 3 individuals hemoglobinuria did not occur until after 420, 1,500, and 810 mg. Hb had been infused, at which times the serum hemoglobin levels had been raised to more than 22, 34, and 35 mg. per cent, respectively. In each instance when hemoglobinuria occurred, it was free Hb, never Hp-Hb. In similar studies of 5 normal individuals, hemoglobinuria did not occur at serum levels of 70, 88, 84, 112, and 65 mg. per cent.

DISCUSSION

The Significance of Hemoglobinemia.—When care has been taken during the collection of blood, healthy persons will be found to have serum which contains very little Hb. In the present survey, all of 26 normal subjects and all but one of 37 other individuals without hematologic disorders have been found to have less than 4 mg. per cent hemoglobinemia. Similar results have been observed by Crosby and Dameshek[13]: in more than 100 normal persons, the range observed was 2 to 5 mg. per cent. The method here employed, in which anticoagulant is not added and in which a greater force of centrifugation is used for a shorter period of time, serves to yield even lower degrees of hemoglobinemia than they encountered. Moreover, the method[14] of Hb measurement used by Crosby and Dameshek has been shown to give spuriously low values at the lower levels of hemoglobinemia.[15] In the method here chosen,[7] the repeatability of determinations is within ± 10 per cent, and recovery of added Hb is 95 per cent. With either method, however, a statistical concept of the limits of normal is needed, since adventitious hemolysis cannot be avoided every time. In this survey values greater than 4 mg. per cent have not been considered normal.

In contrast to normal persons, many individuals with hemolytic disorders are clearly distinguishable because of excessive hemoglobinemia. Indeed, a similar experience has been reported by Crosby and Dameshek,[13] who advise that "definition of the hemolytic syndrome, as it occurs in acquired hemolytic anemia, sickle-cell anemia, and severe Mediterranean anemia . . . be amplified to include a low-grade hemoglobinemia as one of its characteristics." On the other hand, Lathem and Jensen[6] have failed to find basal hemoglobinemia in the plasma of 8 persons with sickle-cell anemia. Such findings would not be expected even in normal persons, so that their failure to have observed any hemoglobinemia must have been due to an insensitive method of detection. The observations made in many of our patients conform to the experiences of Crosby and Dameshek. Others[2] have found hemoglobinemia in pernicious anemia; indeed, early clinicians were aware of the brownish color of the serum in this disorder.[16]

The distinction between adventitiously occurring hemoglobinemia and true in vivo hemoglobinemia is most important, but it cannot be made on the basis of measurement of the degree of hemoglobinemia alone. On the other hand,

this distinction can be made from a consideration of the manner in which this hemoglobin has reacted with the serum. If there is adventitious hemolysis in normal serum, there is found to be only haptoglobin-hemoglobinemia (Hp-Hb), and further study of the serum reveals unbound Hp. On the other hand, in hemolytic disorders with serum hemoglobin levels $>$ 4 mg. per cent, there are other heme pigments besides Hp-Hb, and there is no unbound Hp remaining in the serum.

The Binding of Hemoglobin by Serum Proteins.—In normal persons the Hp proteins present in serum are capable of binding extracorpuscular Hb, and circulating Hb is preferentially bound to these proteins. No other hemoglobin-protein complexes are formed until Hp has been saturated. The term "haptoglobin," as usually referred to in previous studies, has connoted the latent serum binding capacity, i.e., Hp, for additional Hb. A proper recognition of "haptoglobin" already present as Hp-Hb has not been emphasized.

Two combinations of heme pigments with serum proteins other than Hp deserve mention here: (1) methemalbumin, and (2) "haem-binding beta protein." Methemalbumin is a well-defined complex of ferriheme with serum albumin, and the electrophoretic distinction[17] between this and Hp-Hb, as well as specific spectrophotometric analysis,[18] can be easily accomplished. The reality of the other protein, the so-called "haem-binding beta protein" is less clearly established. Neale and associates[3] have found a $beta_1$ globulin which binds Hb after Hp has been saturated. They have discounted the possibility that this $beta_1$ migrating heme pigment could be free Hb A, which under their electrophoretic conditions (pH 8.6) does, indeed, migrate with the speed of $beta_1$ globulin. Nyman[19] has shown a $beta_1$ (at pH 8.6, but $alpha_2$ at pH 7.0) protein which binds heme, but has not shown this to bind hemoglobin, although she has accepted Neale's statement that it does so. Evidence for the existence of these beta globulins has not been appreciated in this study, in which the use of Hb C, rather than Hb A, would have resulted in a better distinction between the presumed "haem-binding beta protein" and free Hb.

The serum haptoglobins have been quantified by modifications of Polonovski and Jayle's[20] original enzymatic method and by several electrophoretic techniques; these various methods[1, 3, 4, 6, 21] have yielded "ranges of normal" values with which the present are in substantial agreement. In Nyman's study of 277 healthy adults, the mean ± 2 S. D. was 28 to 192 mg. per 100 ml.[19]

The Absence of Unbound Haptoglobins in Hemolytic Disorders.—In the great majority of patients with hemolytic disorders, serum Hp is absent: there is no binding of additional Hb. This observation has been amply recorded.[3, 4, 6] In other studies in the literature, however, a satisfactory distinction has not been made between Hp and Hp-Hb when the confusing term "ahaptoglobinemia" has been mentioned, but in each report the absence of haptoglobins has seemingly referred to absent Hp. No comments have been made concerning slight amounts of Hp-Hb, which have occurred not infrequently in this series. Accordingly, it is important to re-emphasize that even persons with hemolytic

diseases may have basal Hp-Hb of low degree despite the lack of free Hp. It is expected that Hp will be absent in active hemolytic disorders, and such has been found in this survey in all except 6 of 37 such persons.

This failure to demonstrate free Hp, though encountered in the great majority of cases, is not fully understood. Laurell and Nyman[22] have clearly shown that a lack of Hp can be brought about in normal persons by the intravenous infusion of Hb, that an Hp-Hb complex is formed and is removed from the blood stream, and that unbound Hp returns to its usual level over a 6 day period. In their 2 subjects and in some of the individuals studied by Lathem and Worley,[17] the clearance of Hp-Hb from plasma has followed a pattern of zero-order kinetics; in general, it has been of the order of 15 to 20 mg. of hemoglobin per 100 ml. plasma per hour clearance. Contrariwise, Garby and Noyes[23] have described a disappearance of minute amounts of radioactively tagged Hb (amounts which should have been bound as Hp-Hb immediately upon entry into

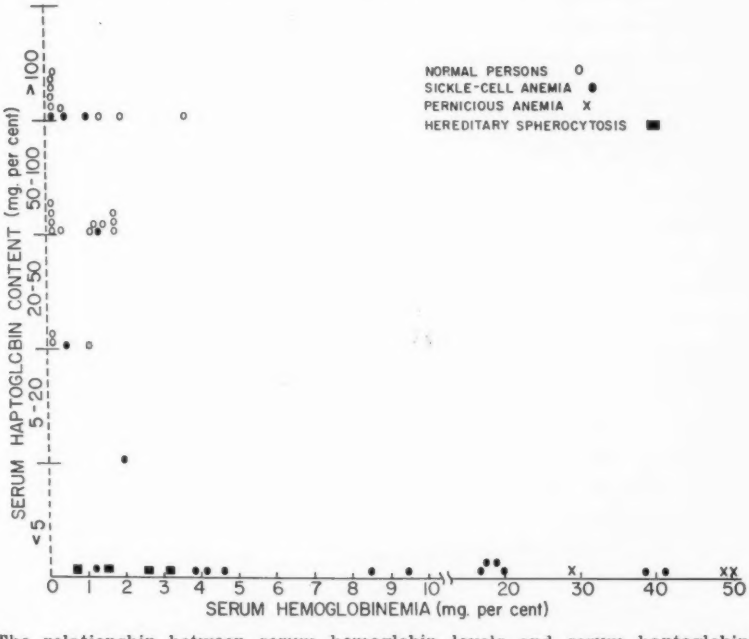

Fig. 3.—The relationship between serum hemoglobin levels and serum haptoglobin levels in selected subjects.

the vascular system) according to an exponential, not a linear, decay. These two findings are not incompatible inasmuch as the various authors were studying different portions of the Hp-Hb disappearance curves, Garby and Noyes having studied the disappearance at more nearly "physiological" concentrations. Whatever the kinetics of clearance of the Hp-Hb may be, it is clear that intravascular hemolysis, either artificially or naturally occurring, serves to exhaust the serum of unbound Hp. Whether such a process of intravascular hemolysis occurs and is the sole reason for depletion of Hp in most persons with hemolytic syndromes is not known. The fact that intravascular hemolysis does

occur in many persons with sickle-cell and pernicious anemia seems undoubted from this survey, although it is quite uncommon in persons with hereditary spherocytosis.

An alternative hypothesis for the absence of Hp in these persons involves inadequate synthesis of these proteins. The presence of Hp-Hb (despite the absence of Hp) in many sicklemics, the occasional finding of Hp in sickle-cell anemia, and the rapid appearance of serum Hp in hereditary spherocytosis after splenectomy and in pernicious anemia patients after therapy conclusively refute this hypothesis as a general explanation.

Regardless of the mechanisms involved, depletion of Hp occurs in hemolytic disorders. Active hemolysis accounts for the lack of free Hp in all except 3 of the individuals studied. In one woman with infectious hepatitis, absence of Hp might have been due either to altered liver synthesis or to the hemolysis which may occur in liver disease.[24] Free Hp has recently been found to be lacking in some healthy individuals presumed not to have hemolytic disturbances. Allison and associates[25] and Giblett[26] have reported such lack in a group of Nigerian and American Negroes, respectively. Hereditary factors have been implicated. In the 2 daughters of Patient D. P. with the sickle trait disorder, such a genetic reason may explain the findings.

The Relationship of Serum Hemoglobin to Serum Haptoglobin.—A priori, the normal red cell envelope is essentially impermeable to Hb and to Hp, else Hp would never be found in serum. Each person with elevated levels of hemoglobinemia has shown no serum Hp. Every individual with normal levels of serum Hp has shown no elevation of serum Hb. This is illustrated in Fig. 3. One sees, however, that there are persons who, despite normal levels of serum Hb, have no free Hp (notably persons with hereditary spherocytosis). Moreover, there are rare individuals, presumed upon clinical and laboratory evidence to have hemolytic syndromes, who yet possess free serum Hp.

The Finding of Serum Haptoglobins in Association With Hemolytic Anemia.—The sera of 6 individuals with hemolytic anemias (5 sickle-cell, 1 hereditary spherocytosis) have had the capacity to bind additional Hb. Is such binding due to Hp? Could it be the "haem-binding beta-protein" of Neale and associates[3] and Nyman[19]? Neither of these authors has shown the hemoglobin binding capacity of these beta globulins, even in sera of hemolytic anemia patients, to be greater than 25 mg. per cent. Each of these 6 showed considerably greater hemoglobin binding. Moreover, the stability of the beta globulin–hemoglobin complex is such that it has not been found in the absence of free Hb. Free Hb has not appeared in these patients along with the presumed Hp-Hb. At pH 7.0, Neale has reported the "beta globulin–hemoglobin complex" to migrate slightly faster than does Hp-Hb. In these 6 individuals, the migration of the hemoglobin complex is the same as that of the complex (Hp-Hb) formed by normal sera. The protein-hemoglobin complex in these individuals is therefore assumed to be Hp-Hb.

A final explanation for the presence of Hp in the serum of such persons with hemolytic states cannot yet be rendered. Does Hp synthesis occur so rapidly in these individuals as to furnish an added amount to the serum? There

is no evidence to arbitrate such a contention. If hemolysis were occurring sporadically, Hp might be found during quiescent periods. The hour-to-hour hemolysis in hemolytic anemias is not fully understood, but the idea that it occurs relatively continuously at a constant rate, from the day-to-day standpoint, is surmised from studies of red cell life spans. Such an explanation for the presence of Hp in these individuals is certainly unproved and quite unlikely. The possibility that hemolysis might be occurring entirely outside the intravascular compartment in these persons seems a plausible hypothesis. (It must be equally true, however, that some persons with the self-same nosologic disorder do have intravascular hemolysis.)

The Concept of Haptoglobin-Hemoglobin Clearance.—Until one has a measurement of the rate of clearance of Hp-Hb from the plasma in vivo for a given individual, it seems unlikely that it would be possible to decide what proportion of hemolysis might be occurring intravascularly and what proportion elsewhere. The rate of clearance of Hp-Hb when Hb has been infused in large quantities (i.e., when the decay rate might logically be expected to approach the limiting maximal rate of zero-order kinetics) seems less germane than a consideration of the rate of clearance of small amounts of Hp-Hb, because the latter more closely parallels the situation obtaining during hemolysis. From a consideration of the rates of clearance of trivial (i.e., levels of approximately 2 mg. per cent) hemoglobinemia in *normal* man, Garby and Noyes[23] state that only a small fraction of Hb is liberated intravascularly. Similar studies of clearance are needed in persons with hemolytic syndromes.

Hemoglobinuria in Hemolytic Anemias.—Previous studies[27] of the pathogenesis of hemoglobinuria in persons with hemolytic disorders have demonstrated that hemoglobinuria occurs more readily in these persons than in healthy persons or persons with nonhemolytic anemias. The probable absence of serum Hp in these persons offers a partial explanation for these results. However, Hp alone cannot determine whether hemoglobinuria will occur. In healthy individuals, Lathem[12] has shown that the average level of free Hb (over and above any protein-bound Hb) is 27 ± 19 mg. per cent before hemoglobinuria occurs. This implies a tubular reabsorption of Hb, but the difference between the filtered load and the urinary Hb recovered is not so great as to prove this implication conclusively.

Sellards and Minot[27] first showed that infusion of Hb causes hemoglobinuria sooner in persons with hemolytic anemias than in those with secondary anemias. More recently, Shen[28] has observed this same difference; however, even in persons with hemolytic anemia, there was no hemoglobinuria unless a serum Hb level of 35 mg. per cent was exceeded. Similar results were observed in 3 individuals—each shown not to have serum Hp by in vitro studies.

A reasonable conclusion is that serum Hp, if present, influences the excretion of Hb in the urine, probably by preventing glomerular filtration. In addition to the Hp effect, there is a renal (tubular?) determinant.

Haptoglobin Physiology.—Much remains to be learned with regard to the genesis of Hp—whence they arise, at what rate, is there reutilization of the same

Hp molecule? What might be the participation of an interstitial pool of Hp? Is there such a factor as intracellular Hp and is this present in macrophages capable of erythrophagocytosis? At what site is Hp-Hb removed from the plasma?

There is no disagreement that Hb binds to Hp in preference to all other serum components and that no other Hb compounds are formed until all the Hp is saturated. Moreover, this binding occurs nearly instantaneously.[29] With the answering of many questions regarding the metabolism of the haptoglobins there may eventuate an understanding of the complexities involved in the destruction of red cells in both the normal and the abnormal situation.

SUMMARY

1. The results of a survey of persons with various hemolytic and nonhemolytic disorders and of normal persons with regard to the presence of serum hemoglobin, the capacity of the serum to bind hemoglobin, and the distribution of hemoglobin within the serum fractions have been reported.

2. Most persons with hemolytic anemias, regardless of hemoglobinemia, have no free haptoglobins, i.e., no capacity to bind additional hemoglobin.

3. Many persons with hemolytic anemia with hemoglobinemia, in spite of no capacity to bind additional hemoglobin, show some hemoglobin already bound to haptoglobin in the native serum. They, therefore, have the ability to synthesize haptoglobins.

4. Rarely persons with hemolytic disorders possess free haptoglobins in their sera. This suggests either different mechanisms, rates, or sites of hemolysis.

5. The question of the clearance of the haptoglobin-hemoglobin complex from serum is aired with regard to deciding between intra- and extravascular hemolysis.

6. In the serum of persons with hemolytic disease, unbound haptoglobins are not present because they are continually depleted. Failure of synthesis does not account for the absence of free haptoglobin.

The assistance of Anne Bjerklie in the performance of the technical aspects of this survey is gratefully acknowledged. I am indebted to Drs. E. Donnall Thomas and W. B. Greenough, III for their valuable suggestions regarding the preparation of the manuscript.

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INFLUENCE OF ALTITUDE CHANGES ON INTESTINAL IRON ABSORPTION

CÉSAR REYNAFARJE, M.D., AND JOSÉ RAMOS, M.D.
LIMA, PERU

Intestinal iron absorption studies have been carried out during changes of altitude with the purpose of clarifying the role that changes of oxygenation as well as the rate of red cell production may play in this phenomenon. The calculation of the amount of ferrous sulfate absorbed was made on the basis of the amount of radioactive iron appearing in the peripheral red cells. In some groups additional studies of the elimination of iron through the feces were made. Intestinal iron absorption increased during the first days the subjects studied were exposed to an altitude of 14,900 feet. It reached a maximum in a week and was less marked one month later. Iron absorption decreased in natives of high altitude brought down to sea level, reached a minimum in 3 weeks, then increased to normal after 16 months. The absorption of iron was essentially the same in natives from high altitude as in sea level subjects when each group was studied in his locality. These findings suggest that a greater or smaller bone marrow demand associated with changing red cell production is the stimulus for the intestinal absorption of iron related to altitude changes rather than oxygen saturation of the blood or oxygen tension at the intestinal level.

THE regulating mechanisms of intestinal iron absorption are still not very well known. It has been suggested that hypoxemia, acting on the intestinal mucosa, plays an important role in this phenomenon.[1] On the other hand, it has been found that an increase in the absorption of this metal occurs when there is a greater demand due to hyperactivity of erythropoiesis, while a decrease occurs when there is a depression in the red blood cell formation.[2] These observations suggested the applicability of the performance of iron absorption studies during changes of altitude, both in subjects taken from sea level to an altitude of 14,900 feet, and in natives of that altitude taken down to sea level. Such changes cause variations in oxygenation and, consequently, in the rate of red cell production.[3]

MATERIALS AND METHODS

Eighty-two healthy adult men were studied. They were divided into 14 groups, each of which was investigated under different conditions of altitude, as will be explained. Most of the groups consisted of 5 subjects each.

The iron was given orally as ferrous sulfate tagged with Fe^{59}.[4] In the majority of the experiments a 100 mg. dose of metallic iron was administered which was equivalent to

From the Institute of Andean Biology and the Department of Pathological Physiology, Faculty of Medicine, University of San Marcos, Lima, Peru.

Supported by Contract No. AF 41 (657)-249 from the School of Aviation Medicine, United States Air Force.

Received for publication Oct. 5, 1960.

1.5 to 1.8 mg. per kilogram of body weight. This relatively high dose was selected because it provided smaller individual variations in the amount absorbed than did the lower doses. However, in 2 groups, smaller quantities were administered, i.e., 30 mg. metallic iron, equivalent to 0.40 to 0.50 mg. per kilogram of body weight. In all cases the iron was taken by subjects in a fasting state. Eight days later consecutive blood samples were taken until a maximum of radioactivity was obtained. A scintillation counter was used to measure radioactivity in the red blood cells. In 10 of the 14 groups the percentage absorbed was calculated on the basis of the maximum amount of labeled iron appearing in the R.B.C. In the 4 other groups the fraction eliminated was measured in addition to that which appeared in the red cells. To determine the amount of unabsorbed iron, a careful collection of stools was made for 8 to 10 days. Following the technique of Bothwell[2] the entire stool sample of each subject was treated with .5 L. sulfuric acid for 10 days with periodic stirring. At the end of that time the mixture was strongly agitated, a 2 ml. sample removed, and the radioactivity measured. The uniformity of the counts obtained in 2 or 3 samples from the same subject indicated that there had been an even distribution of iron in the mixture.

TABLE I. INTESTINAL IRON ABSORPTION FOLLOWING ASCENT TO HIGH ALTITUDE (PERCENTAGE OF 100 MG. DOSE)

	SEA LEVEL SUBJECTS			
	AT LIMA (SEA LEVEL)	AFTER ASCENT TO MOROCOCHA (14,900 FEET)		
		2 DAYS	8 DAYS	30 DAYS
Fe appearing in R.B.C. (Mean ± S. D.)	3.6 ± 1.27	14.3 ± 3.78	16.3 ± 2.34	7.2 ± 3.10
Fe reaching circulating plasma but not R.B.C.*	0.5	0	0	0
Total	4.1	14.3	16.3	7.5
Fe uptake for Hb synthesis*	86/100	100/100	100/100	95/100
Hemoglobin (grams %)	15.1	15.9	16.2	17.2
Number of subjects studied	15	10	5	5

*Estimated on basis of previous studies.

RESULTS

Absorption After Ascent to 14,900 Feet.—In Table I and Fig. 1 the data obtained in 3 groups of subjects taken from sea level to Morococha (14,900 feet) are presented. These 3 groups were studied 2, 8, and 30 days after their arrival, respectively. These results were compared with those obtained at sea level with a group of 15 subjects born there. A 100 mg. iron dose was given to each subject. The per cent absorption was calculated on the basis of the iron that appeared in the red cells, as mentioned before, but a correction factor was added for that fraction of the absorbed iron that presumably had entered the circulating plasma but which did not appear in the red cells. This factor was applied on the basis of previous studies of intravenous iron uptake carried out in subjects under the same conditions of exposure to high altitude. By this correction we have replaced the double isotope method described by Saylor and Finch[5] which is used for the same purpose in cases in which the intravenous iron uptake is unknown.

In the group of 15 native sea level subjects studied under normal conditions of oxygen tension, the average per cent of absorbed iron that appeared in the red cells was 3.6, with a standard deviation of ± 1.27. This figure represents 86 per cent of the absorbed iron that reached the circulating plasma, of which the total was estimated to be 4.1 per cent.

Two days after their ascent to 14,900 feet, a group of 10 subjects showed a much greater absorption of iron, as reflected by the appearance of 14.3 ± 3.8 per cent in the erythrocytes. In the group studied after 8 days the absorption was even higher, being 16.3 ± 2.3 per cent. In both groups it was assumed that all absorbed iron was used in the production of red cells, in accordance with previous investigations in which we have demonstrated that the total amount of iron available in the circulating plasma was utilized for hemoglobin synthesis during this early stage of adaptation to high altitude.[3] In the group of 5 subjects studied 1 month (30 days) after arrival at high altitude, a tendency was observed toward a return to normal iron absorption. An average of 7.2 ± 3.10 per cent of iron appeared in the red blood cells in this group, and it was assumed that this represented 90 per cent of the 7.9 per cent which reached the plasma.

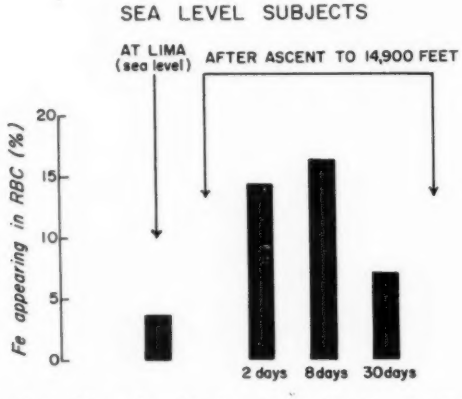

Fig. 1.—Intestinal iron absorption of subjects at sea level and after their ascent to 14,900 feet. Each column represents the mean value of Fe appearing in R.B.C.

Absorption After Descent From High Altitude.—This study was performed in 5 groups of natives of Morococha (14,900 feet) who were observed at 2 days, 1 week, 3 weeks, 2 months, and 16 months after descent to sea level. Base line values were taken from a study in Morococha of natives of the city. The iron dose was 100 mg.

It can be seen in Table II and Fig. 2 that the mean value for absorbed iron appearing in the red cells of 8 natives of high altitude studied in their place of origin was 4.8 ± 1.2 per cent, which when corrected becomes 5.2 per cent. This value is slightly higher than, but not significantly different from, values obtained in sea level subjects studied under conditions of normal oxygenation.

Regarding the natives of high altitude brought down to sea level, it can be seen that in the group of subjects studied 2 days after their arrival there was considerable decrease in iron absorption. The mean value in this group for the iron appearing in the red cells was 1.48 ± 0.57 per cent. The decrease was slightly more marked in the 8 day group (1.25 ± 0.38 per cent), and reached a minimum of 0.75 ± 0.16 per cent in the group studied 3 weeks after departure from high altitude. When the correction factor for the observed iron reaching

the circulating plasma but not appearing in the red blood cells is added, the above values became 2.46, 2.11, and 1.20 per cent, respectively, which represents an increment of 60 per cent over the former values. In the group studied 2 months after arrival at sea level there was a tendency toward a recovery of normal iron absorption (2.7 ± 1.1 per cent), and the 16 month group had returned nearly to normal absorption, with a mean of 3.6 per cent, corrected for iron not appearing in the red cells.

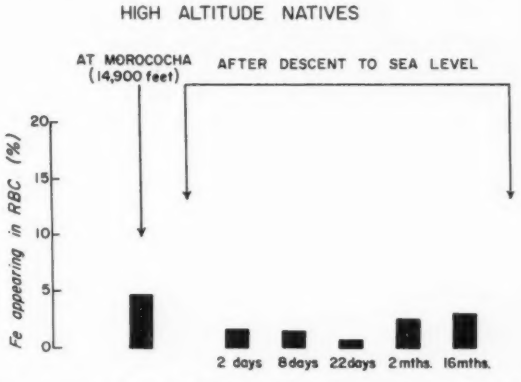

Fig. 2.—Intestinal iron absorption of subjects at high altitude and after their descent to sea level. Each column represents the mean value of Fe appearing in R.B.C.

TABLE II. INTESTINAL IRON ABSORPTION FOLLOWING DESCENT FROM HIGH ALTITUDE TO SEA LEVEL (PERCENTAGE OF 100 MG. DOSE)

	HIGH ALTITUDE SUBJECTS					
		AFTER DESCENT TO SEA LEVEL (LIMA)				
	AT MOROCOCHA (14,900 FEET)	2 DAYS	8 DAYS	22 DAYS	2 MONTHS	16 MONTHS
Fe appearing in R.B.C. (Mean ± S. D.)	4.80 ±1.20	1.48 ±0.57	1.25 ±0.38	0.75 ±0.16	2.70 ±1.1	3.10 ±0.86
Fe reaching circulating plasma but not R.B.C.*	0.40	0.98	0.86	0.50	0.44	0.50
Total	5.20	2.46	2.11	1.25	3.14	3.60
Fe uptake for Hb synthesis*	92/100	60/100	60/100	60/100	86/100	86/100
Hemoglobin (grams %)	20.1	19.0	18.4	14.1	13.8	13.8
Number of subjects studied	8	4	5	5	5	5

*Estimated on basis of previous studies.

Effect of Erythropoietic Stimulation After Iron Absorption.—In the group of native subjects studied 2 days following their arrival at sea level from high altitude it was noted, as indicated, that only a small percentage of labeled iron appeared in the red cells. The possibility that this might be actually only a small part of the total iron absorbed was investigated in the group, on the basis that the fraction not incorporated in the cells might form a reserve (''labile pool'') which could be used in later demands of the bone marrow, as has been described in cases of pernicious anemia.[6] For this purpose these subjects were exposed to further erythropoietic stimulation by a return to high altitude immediately after maximum radioactivity in red cells had been reached at sea level (11 days). Two weeks after their return to high altitude, and without further

administration of iron, blood samples were taken in which no additional labeled iron was found. Table III shows the percentages of radioactivity in blood samples before and after the return of these subjects to high altitude.

Recovery of Iron in Feces.—In a first series of experiments 10 students born at sea level were studied, 5 at Morococha (14,900 feet) and 5 at sea level. Thirty milligrams of labeled iron as ferrous sulfate was given to each one. This small dose was used in order to obtain a greater percentage uptake, thus permitting better analysis of the difference between that iron appearing in the red cells and that eliminated in the feces. The subjects studied at high altitude took the iron 3 days after their arrival.

TABLE III. IRON ABSORPTION IN HIGH ALTITUDE NATIVES AT SEA LEVEL AND UPON RETURN TO HIGH ALTITUDE (PERCENTAGE OF Fe APPEARING IN R.B.C.)

SUBJECT	SEA LEVEL	HIGH ALTITUDE
1	0.71	0.69
2	1.10	1.30
3	1.39	1.20
4	1.80	1.70
Mean	1.25	1.22

TABLE IV. OBSERVATIONS ON FECAL ELIMINATION OF NONABSORBED IRON AT SEA LEVEL AND 3 DAYS AFTER ARRIVAL AT MOROCOCHA (14,900 FEET) (30 MG. DOSE)

SUBJECT	IRON APPEARING IN R.B.C. (%)	IRON REACHING PLASMA BUT NOT R.B.C. (ESTIMATED) (%)	IRON ELIMINATED IN FECES (%)	TOTAL IRON (%)
At sea level (Lima)				
1	2.1	0.4	96.3	98.8
2	7.0	1.3	88.2	96.5
3	7.0	1.3	90.2	98.5
4	10.2	1.7	90.7	102.6
5	2.7	0.5	92.6	95.8
Mean ± S. D.	5.8 ±3.0	1.0	91.6 ±2.6	98.4
After arrival at Morococha				
1	15.7	0	83.2	98.9
2	15.3	0	83.5	98.8
3	23.4	0	75.4	98.8
4	12.1	0	79.8	91.9
5	31.3	0	68.6	99.9
Mean ± S. D.	19.6 ±6.9	0	78.1 ±5.6	97.7

Individual data are presented in Table IV. The average value of iron recovered in feces of the group studied at high altitude was 78.1 ± 5.5 per cent, and in the group studied at sea level it was 91.6 ± 2.8 per cent. Absorbed iron appearing in the red cells, plus that which was assumed to have reached the circulating plasma, but which was not incorporated into hemoglobin, averaged 19.6 ± 6.9 per cent in the group observed at high altitude and 6.8 ± 3.5 per cent in that studied at sea level. The total of the iron recovered in the feces plus the amount of iron absorbed in the blood had a mean value of 97.7 per cent at high altitude and 98.4 per cent at sea level.

The second series of studied of elimination of nonabsorbed iron was carried out in a group of 4 natives of high altitude 8 days after their arrival in Lima (sea level). For comparative purposes the same type of observation was done at sea level in 5 subjects native to the area. To both groups 100 mg. of iron was administered as ferrous sulfate. The average value for iron recovered in feces was 96.0 ± 2.5 per cent in the natives of high altitude brought down to sea level (Table V). Addition of the absorbed iron found in blood already corrected gave a total of 98.1 per cent. In the group of sea level subjects studied in Lima the iron found in feces was 93.5 ± 2.7 per cent, which, when added to the absorbed iron in blood (corrected), totaled 97.4 per cent.

TABLE V. OBSERVATIONS ON FECAL ELIMINATION OF NONABSORBED IRON IN NATIVES OF HIGH ALTITUDE 8 DAYS AFTER ARRIVAL AT SEA LEVEL IN COMPARISON WITH NORMAL SUBJECTS IN LIMA (SEA LEVEL) (100 MG. DOSE)

SUBJECT	IRON APPEARING IN R.B.C. (%)	IRON REACHING PLASMA BUT NOT R.B.C. (ESTIMATED) (%)	IRON ELIMINATED IN FECES (%)	TOTAL IRON (%)
Normal subjects at sea level				
1	4.1	0.6	95.0	99.6
2	3.5	0.5	93.7	97.7
3	4.0	0.6	89.6	94.2
4	1.5	0.2	96.8	98.5
5	4.0	0.5	92.2	96.7
Mean ±	3.4	0.5	93.5	97.4
S. D.	±1.1		±2.7	
Morococha natives 8 days after arrival at Lima				
1	1.1	0.7	98.2	100.0
2	1.8	1.2	92.1	95.1
3	1.3	0.9	95.5	97.7
4	0.9	0.6	98.0	99.5
Mean ±	1.3	0.8	96.0	98.1
S. D.	±0.3		±2.5	

In the four groups just described there were individual variations in the total amount of iron recovered in blood and feces. In the majority of the cases this total was below 100 per cent of the given dose. Our findings are, however, similar to those of Bothwell,[2] whose technique was used.

DISCUSSION

The observations presented here indicate that changes in altitude produce modifications in iron absorption. These variations are in close relationship with the demand for this element in connection with erythropoietic activity. Iron absorption was increased 3 to 4 times during early exposure of the subjects to high altitude, a period during which a marked hyperactivity of the red blood cell precursors has been demonstrated.[3] On the other hand, there was a decrease in iron absorption during descent from high altitudes, coincidental with a depression of bone marrow activity.[3, 7] However, permanent residents of high altitude in whom equilibrium of erythropoietic activity was demonstrated previously[3, 8, 9] did not show a significant difference in iron absorption as compared with results from people born and living at sea level. These observations

suggest that greater or smaller bone marrow demand, associated with changing red cell production, is the stimulus for the intestinal absorption of iron related to altitude changes rather than oxygen saturation of the blood or oxygen pressure at the intestinal level. Only in this way can one explain the finding of no significant elevation of iron absorption in natives of high altitude studied in their locality (14,900 feet) despite their arterial oxygen saturation of 80 per cent. The close relationship between the level of red cell production and iron absorption suggests that the regulating mechanisms of iron absorption during altitude changes must take place in intimate relation to or through a mechanism similar to that controlling erythropoiesis which is now generally believed not to be brought about by direct action of the changes in oxygen pressure or content on the erythropoietic tissues.[10-14]

The fact that there was no increase in the appearance of previously administered labeled iron in the red cells of high altitude residents brought to sea level and then returned to high altitude indicates that the iron absorbed in these subjects did not take part in the "labile pool."[15, 16] In other words, all the absorbed iron went either for hemoglobin formation or for the fulfillment of other biologic needs.

The studies of fecal recovery of nonabsorbed iron confirm the modifications of iron absorption during changes of altitude which were interpreted on the basis of the quantity incorporated in red cells plus the small amount presumed to reach the circulation but not utilized in hemoglobin synthesis. It is necessary, however, to make individual analyses of these determinations. The total amount of iron in feces and blood (corrected) was in nearly all cases less than 100 per cent of the given dose. In some subjects the amount of unrecovered iron was as high as 10 per cent. This could be attributed to incomplete collection of feces, but, assuming thorough collection, it may still be accounted for by the removal of the remaining fraction by the liver during primary passage through that organ by the portal circulation, and its storage, probably in the form of hemosiderin, which is difficult to mobilize. This phenomenon may occur in subjects who have low iron reserves, but there may be some other unknown causes.

Finally, it should be pointed out that the stimulation or depression of red cell production due to altitude changes offers a fertile field for studies on the mechanisms of iron absorption.

SUMMARY AND CONCLUSIONS

The absorption of iron in the form of ferrous sulfate has been studied in various conditions of altitude exposure in 82 subjects. From the observations made the following conclusions can be deduced:

1. Intestinal absorption of iron increased during the first days of exposure of the subjects to an altitude of 14,900 feet. It reached a maximum in a week, and was less marked 4 weeks later.

2. Iron absorption decreased in natives of high altitude who were brought down to sea level, reached a minimum in 3 weeks, then increased to normal at 16 months.

3. Iron absorption was essentially the same in natives from high altitude as in sea level subjects when each was studied in his locality. The slightly higher values reached in subjects from a high altitude lacked statistical significance.

4. Studies of the recovery of iron in feces confirmed the changes that were interpreted on the basis of the amount of iron appearing in the red blood cells plus the iron that presumably reached the circulating plasma but was not used in red cell formation. The possible significance of the failure to recover a fraction of the total iron in some cases is discussed.

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HEPATIC DYSFUNCTION AND ABNORMALITIES OF THE SERUM PROTEINS AND SERUM ENZYMES IN SICKLE-CELL ANEMIA

G. Hilkovitz, M.B., B.Ch.,* and A. Jacobson, B.A.**
Richmond, Va.

Detailed reports concerning hepatic dysfunction in sickle-cell anemia have not been published since 1953. Information about serum enzymes or serum proteins in this disease is either lacking or conflicting. Furthermore, in the light of modern tests, the diagnosis of sickle-cell anemia previously was based upon evidence insufficient to distinguish it clearly from the other sickle-cell diseases.

In the present study, hepatic function, activity of the serum enzymes, and composition of the serum proteins were evaluated in 14 patients with sickle-cell anemia, in whom the diagnosis was confirmed by filter-paper and starch-gel electrophoresis and alkali denaturation of hemoglobin.

Hepatic dysfunction, unrelated to the severity of anemia, was present in all patients, including the younger and relatively asymptomatic cases. The serum enzymes were normal in only one patient and the serum proteins in two. Conclusions, different from those of some earlier investigators, were reached concerning the albumin/globulin ratio and prothrombin concentration. Among all the investigations made, the only significant correlation was an inverse relationship between hemoglobin concentration and urinary coproporphyrin excretion.

CLINICAL, biopsy, and autopsy studies in sickle-cell anemia have shown that the liver frequently is enlarged and is the seat of pathologic changes.[1] Hepatic function in sickle-cell anemia was evaluated by Green and associates[2] in 1953; in 1955 Bogoch and co-workers[3] described biochemical, histologic, and histochemical observations on the liver of 4 patients whose diagnosis of sickle-cell anemia was based upon positive sickling tests without hemoglobin electrophoresis. Reports have been published on serum alkaline phosphatase activity[4] and the serum proteins[5, 6] in sickle-cell anemia, but little is known concerning serum glutamic oxalacetic and pyruvic transaminases and serum lactic dehydrogenase in the disease.

The purpose of the present investigation was an evaluation of liver function in sickle-cell anemia by employment of a variety of tests different from or greater than those used in earlier studies, and, at the same time, analysis of the serum proteins by electrophoresis and measurement of the activity of serum enzymes.

From the Laboratory for Clinical Investigation, Medical College of Virginia, Richmond, Va.

Received for publication April 28, 1960.

*A. D. Williams Post-doctoral Research Fellow, Laboratory for Clinical Investigation, Medical College of Virginia, Richmond, Va.

**A. D. Williams Pre-doctoral Research Fellow, Laboratory for Clinical Investigation, Medical College of Virginia, Richmond, Va., and senior medical student, Northwestern University Medical School, Chicago, Ill.

PATIENTS AND METHODS

Fourteen patients were studied in whom the diagnosis of sickle-cell anemia was confirmed by filter paper and starch gel electrophoresis, and alkali denaturation of hemoglobin (Table I). The patients were examined in the order in which they appeared at an outpatient clinic or in the hospital wards so that there was no selection of patients on the basis of unusually marked jaundice or hepatic enlargement.

TABLE I. FILTER PAPER AND STARCH GEL ELECTROPHORESIS, AND ALKALI DENATURATION OF HEMOGLOBIN

CASE NO.	AGE (IN YEARS)	SEX	SEALED-SLIDE TEST FOR SICKLING	FILTER PAPER ELECTROPHORESIS (HB.)	ALKALI DENATURATION (HB. F %)	STARCH GEL ELECTROPHORESIS (HB. A_2 %)*
Group A.—						
1	7	F	Positive	S	5.2	3.1
2	7	M	Positive	S	2.7	4.2
3	8	M	Positive	S	5.5	
4	13	M	Positive	S	2.0	
5	13	M	Positive	S	2.4	2.6
6	14	F	Positive	S	5.4	2.6
7	16	F	Positive	S	3.4	4.2
8	38	F	Positive	S	6.9	3.3
9	28	M	Positive	S	2.0	3.4
10†	27	F	Positive	S	4.1	3.7
Group B.—						
11†	27	F	Positive	S	4.1	3.7
12	19	F	Positive	S	3.4	6.0‡
13	29	M	Positive	S	4.2	4.7
Group C.—						
14	11	M	Positive	S	12.4	3.8
15	13	F	Positive	S	4.4	3.5

*Values for hemoglobin A_2 in sickle-cell anemia = 1.9 per cent to 4.7 per cent; in thalassemia trait, 5.2 per cent to 8.4 per cent.

†Cases Nos. 10 and 11 are the same patient.

‡Probable diagnosis: sickle-cell thalassemia.

Ten subjects (Group A) were relatively asymptomatic when tested; the only complaints were of moderate lassitude and dyspnea on exertion. Icterus was mild and fever absent, but the liver was palpable in 70 per cent of these patients (Table II). Three patients (Group B) were hospitalized: two with severe bone pains, fever, mild icterus, and no hepatomegaly (Cases 11 and 12, Table II) and one with generalized abdominal discomfort, anorexia, fever, marked icterus, and an enlarged, tender liver (Case 13, Table II). Two patients (Group C) were confined to the hospital because active tuberculosis complicated sickle-cell anemia, but hepatomegaly was absent and jaundice mild. One patient was studied during an asymptomatic phase (Case 10, Group A) and again while she had bone pains and fever (Case 11, Group B).

Each patient was observed for 4 consecutive days while stools and urine were collected for quantitative estimation of fecal and urinary urobilinogen[7] and urinary coproporphyrins.[8] Blood was drawn on the first day for enumeration of reticulocytes[9] and measurement of hemoglobin concentration[9] serum bilirubin,[10] plasma prothrombin concentration,[11] serum vitamin B_{12},[12] serum albumin and globulin,[13] serum alkaline phosphatase,[14] serum glutamic oxalacetic transaminase (SGOT),[15] serum glutamic pyruvic transaminase (SGPT),[15] serum lactic dehydrogenase (SLD),[16] and serum pseudo-cholinesterase.[17] On the first day bromsulphalein (BSP) retention was measured[18] and electrophoresis of serum proteins was performed.[19] When possible, reticulocyte counts and estimations of hemoglobin concentration, serum bilirubin, plasma prothrombin concentration, serum alkaline phosphatase, thymol turbidity and flocculation, and cephalin flocculation were repeated on the fourth day.

TABLE II. STUDIES OF HEPATIC FUNCTION IN 14 PATIENTS WITH SICKLE-CELL ANEMIA

GROUP	CASE NO.	AGE (IN YEARS) AND SEX	LIVER SIZE (CM. BELOW COSTAL MARGIN)	HB. (GM. %)	RETICULOCYTES (%)	UROBILINOGEN (MG./24 HOURS)		URINARY COPROPORPHYRINS (γ GM./24 HOURS)	SERUM BILIRUBIN (MG. %)		PROTHROMBIN CONCENTRATION (%)	SERUM B_{12} (γ γ GM./ML.)	BROMSULPHALEIN RETENTION (%)	ALKALINE PHOSPHATASE (BESSEY-LOWRY UNITS)	THYMOL TURBIDITY (MACLAGEN UNITS)	CEPHALIN FLOCCULATION	THYMOL FLOCCULATION	SERUM PSEUDO-CHOLINESTERASE (Δ PH UNITS/HOUR)	SERUM PROTEINS		
						URINE	FECES		DIRECT	INDIRECT									ALBUMIN (GM. %)	GLOBULIN (GM. %)	ALBUMIN: GLOBULIN
Normal					2	0-4	Up to 280	M 300 F 275	Up to 0.4	Up to 0.7	50–100	Up to 800	Up to 5%	A. 2.9* C. 8.0*	Up to 4	1+	0	0.5	3.4–4.5	2.5–3.6	1.4–2.5
A (A-symptomatic)	1	7 F	1	8.1 9.0	40	19	805	240	0.5 0.3	5.8 3.0	43 —	645 —	— —	4.5 4.6	1 2	0 —	0 0	0.57 —	4.8 —	2.6 —	1.84
	2	7 M	1	7.2	14	—	—	—	0.3	2.0	50	415	—	3.5	5	4	2	0.57	4.0	3.7	1.08
	3	8 M	4	— 8.1	—	22	705	525	0.3	2.3	60	460	—	4.2	1.0	2	0	0.76	4.5	3.4	1.32
	4	13 M	0	6.5 6.7	36 34	34	955	620	0.7 0.7	5.8 6.0	50 43	570 —	7.6 —	4.6 6.2	3 4	1 1	0 1	0.56 —	3.9 —	4.1 —	0.95
	5	13 M	1	4.9 4.2	42 29	1	1,450	1,280	0.5 0.6	4.6 4.4	50 60	820 —	4.8 —	6.0 6.1	7 4	3 3	0 0	0.53 —	3.8 —	4.1 —	0.92
	6	14 F	2	6.5 6.7	10 11	21	265	665	5.1 3.7	7.8 6.4	33 43	1,230 —	33.1 13†	6.7 6.5	10.0 10.0	4 4	2 3	— 0.36	— 2.4	6.4	0.37
	7	16 F	0	7.6 8.7	20 28	56	740	490	0.4 0.5	5.1 6.3	38 50	635 —	— 8.2	4.4 6.0	4 5	2 2	0 0	0.48 —	4.4 —	3.3 —	1.33
	8	38 F	1	8.5 7.8	16 20	29	160	170	0.4 0.4	2.6 2.9	43 38	365 —	— 2.3	1.3 1.4	2 3	2 2	0 —	0.64 —	4.8 —	3.7 —	1.29
	9	28 M	2	6.3 5.8	35 70	468	1,795	935	2.6 —	9.2 —	60 43	325 —	13.3 1†	4.0 7.6	— 3	3 —	— 0	0.75 —	4.3 —	3.5 —	1.23
	10‡	27 F	0	8.3 8.5	13 —	19	340	430	1.0 0.9	3.1 2.1	60 43	— 755	28.1 —	4.7 6.3	12 13.0	4 4	2 3	0.28 —	3.0 —	5.5 —	0.54
B (Symptomatic)	11‡	27 F	0	8.3 6.5	9 7	63	—	465	0.7 0.3	3.0 1.1	— 50	— 390	17.2 —	3.5 2.2	4 —	— —	2 —	0.49 —	2.7 —	4.5 —	0.6
	12	19 F	0	9.4	8	—	—	—	0.4	2.6	38	735	8.5	1.8	1.2	2	0	0.67	4.6	2.8	1.64
	13§	29 M	8	8.5 11.4 11.2	24 13 1	— — 13	— — 380‖	— — 755	17.9 7.1 4.6	18.9 9.8 8.2	30 27 38	— 2,565	— — 12.6 –5†	8.5 9.9 6.4	1.7 1.6 3.0	1 0 —	— 0 —	— 0.49 —	— 3.3 —	— 4.2 —	0.78
C (Active tuberculosis)	14	11 M	0	8.7 8.4	6 —	31	130‖	130	0.4 0.3	3.5 2.8	38 38	585 —	— 2.5	3.5 3.2	1.5 —	2 2	0 —	0.52 —	— —	— 3.9	1.0
	15§	13 F	0	8.1 7.6	20 24	— 11	— 690‖	— 295	0.5 0.4	4.6 4.8	43 60	505 —	— 2.3	5.7 6.5	1.9 2.0	2 2	0 0	0.71 —	4.2 —	3.2 —	1.31

*A. = adult; C. = child.
†Corrected BSP: Zieve and associates.[18]
‡Cases Nos. 10 and 11 were the same patient.
§Received blood transfusions.
‖Received antibiotics.

RESULTS

The results of hepatic function tests are given in Table II; the values obtained for serum enzymes and proteins appear in Table III; the incidence of abnormal results obtained among the asymptomatic patients, is given in Table IV.

DISCUSSION

There was evidence of hepatic dysfunction in all the patients studied, even in those at the relatively young age of 7 years and in cases where acute clinical manifestations of sickle-cell anemia were absent. The degree of hepatic dysfunction was not related to the severity of the anemia, nor was it always greater in the older subjects in the series. The results indicate that hepatic dysfunction is present so frequently in sickle-cell anemia that its discovery need not occasion surprise.

Individual tests are discussed below in order of the frequency with which they were abnormal among the asymptomic patients.

Urinary Urobilinogen.—Factors augmenting urobilinogenuria in sickle-cell anemia are as follows: hemolysis, liver disease,[20] and tissue infarction.[21] Urobilinogenuria was excessive in all patients and was not related to hemoglobin concentration. It was discovered that Patient No. 5, who excreted less than 1 mg. per day during the test period, had erred in collection of the urine; two weeks earlier he had excreted 35 mg. per day.

Green and associates[2] had found, with the use of a semiquantitative technique, that urinary urobilinogen was always high in patients with marked jaundice, bilirubinuria, and elevated serum alkaline phosphatase activity. A direct relationship could not be found in the present study between the serum bilirubin ("direct," "indirect," or "total") and the amount of urobilinogen in the urine.

Since increased quantities of fecal urobilinogen were also detected, extrahepatic biliary obstruction was probably not a factor contributing toward the jaundice. Although two patients in Group A, who were severely constipated, excreted normal amounts of fecal urobilinogen during the 4 day test period, calculation of fecal urobilinogen per 100 Gm. of feces gave values in excess of normal. In another patient (Case 14, Group C) low fecal urobilinogen was probably the result of antibiotic treatment. There was no relationship between fecal urobilinogen and hemoglobin concentration or serum bilirubin levels.

Serum Alkaline Phosphatase.—Abnormalities were confined to patients over the age of 16 years. SGOT activity was increased, without exception, in the same patients, indicating, perhaps, that liver damage, rather than bone disease, caused the abnormality in alkaline phosphatase.[22] It could not be determined from earlier reports[3, 4] whether increased serum alkaline phosphatase activity in sickle-cell anemia was due to bone disease or to intrahepatic biliary obstruction. Green and associates,[2] having found normal levels of serum calcium and phosphorus, concluded that bone lesions in sickle-cell anemia probably did not increase serum alkaline phosphatase activity.

bercu-
losis)

§Received blood transfusions.
‖Received antibiotics.

*A. = adult; C. = child.
†Corrected BSP; Zieve and associates.[38]

TABLE III. ACTIVITY OF SERUM ENZYMES AND ELECTROPHORETIC ANALYSIS OF SERUM PROTEINS IN SICKLE-CELL ANEMIA

GROUP	CASE NO.	AGE (IN YEARS) AND SEX	HB. (GM. %)	SGOT (S-F UNITS)	SGPT (S-F UNITS)	SLD (B-B UNITS)	SERUM ELECTROPHORESIS				
							GLOBULIN				ALBUMIN (%)
							α 1 (%)	α 2 (%)	β (%)	γ (%)	
Normal			M 14 to 18 F 12 to 16	8-40	5-35	550	5 to 8	7 to 12	9 to 16	11 to 20	49 to 62
A	1	7 F	8.1	46	13	960	8	6	12	16	58
(Asymptomatic)	2	7 M	7.2	30	16	580	6	6	14	27	48
	3	8 M	8.1	24	8	480	7	8	14	17	54
	4	13 M	6.5	70	23	800	6	7	18	28	41
	5	13 M	4.9	58	21	680	6	7	16	24	46
	6	14 F	6.7	400	50	700	5	6	15	52	22
	7	16 F	7.6	30	8	560	6	7	13	20	53
	8	38 F	8.5	28	12	720	9	5	16	27	43
	9	28 M	6.3	58	13	1,260	8	7	17	19	49
	10*	27 F	8.4	116	140	540	7	4	16	42	31
B	11*	27 F	7.4	62	23	1,080	6	8	11	41	34
(Symptomatic)	12	19 F	9.4	28	8	1,100	9	10	13	12	57
	13†	29 M	11.4‡	98	113	1,160	8	9	25	26	32
C	14†	11 M	8.7	58	14	680	7	7	16	25	45
(Active tubercu-			8.4	44	—	680	6	8	19	19	48
losis)	++15†	13 F	8.1	50	12	1,160	9	10	21	14	46

*Cases Nos. 10 and 11 are the same patient.
†Received antibiotics.
‡Received blood transfusion.

"Direct" Serum Bilirubin.—This fraction of the serum bilirubin increases as the result of parenchymal liver damage or biliary obstruction, but there was no evidence of the latter among the patients investigated in spite of the reported increased incidence of gallstones in sickle-cell anemia.[2, 23]

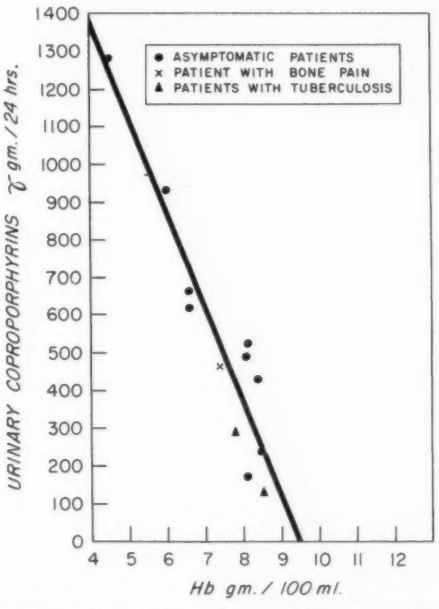

Fig. 1.—The inverse relationship between hemoglobin concentration and urinary coproporphyrin excretion.

BSP Retention.—Bogoch and co-workers[3] found increased BSP retention in 3 out of 4 patients. In the present study, BSP retention was calculated with the use of the correction factor of Zieve and associates[18] for icteric patients; in Patient No. 13 this calculation gave a result so low that it was inconsistent with the clinical and laboratory evidence of acute hepatic disturbance.

Prothrombin Concentration.—The results were comparable to those obtained by Green and co-workers[2] but contrasted with the findings of Walters,[24] who reported that "the plasma prothrombin concentration was increased in ten out of twelve patients" when he compared their plasma with a pooled plasma from normal subjects.

Cephalin Flocculation, Thymol Turbidity, and Thymol Flocculation.—These are considered together for convenience. It was remarkable that all 3 tests were normal in a patient (No. 13) with jaundice, hepatomegaly, fever, hyperglobulinemia, hypoalbuminemia, bilirubinemia, and elevated serum levels of alkaline phosphatase and vitamin B_{12}. It is noteworthy that Bogoch and associates[3] found no correlation between disturbances of the serum proteins and abnormalities in the turbidity and flocculation tests.

Serum Pseudo-Cholinesterase.—Marked or sustained reduction in the activity of this enzyme, associated frequently with hypoalbuminemia (Patients 6, 8, 12, and 13), suggests severe hepatic dysfunction. In Patient No. 7, however, decreased activity of the enzyme was not accompanied by hypoalbuminemia.

TABLE IV. INCIDENCE OF ABNORMAL TESTS AMONG RELATIVELY ASYMPTOMATIC PATIENTS WITH SICKLE-CELL ANEMIA

DETERMINATION	NUMBER OF PATIENTS	NUMBER OF DETERMINATIONS	MEAN	S.D.	MEDIAN	NORMAL VALUES	NUMBER OF DETERMINATIONS ABNORMAL	% OF DETERMINATIONS ABNORMAL
Urobilinogen (urinary) (mg./24 hours)	9	9	74	140	22	0 to 4	(> 4.0) 8	89
SLD (B-B units)	10	10	730	220	680 to 720	550	(> 550) 8	80
Alkaline phosphatase (adults) (B-L units)	4	8	4.5	2.1	4.4 and 4.7	0.8 to 2.9	(> 2.9) 6	75
Serum bilirubin (direct) (mg. %)	10	17	1.6	1.5	0.5	0.1 to 0.4	(> 0.4) 11	65
SGOT (S-F units)	10	10	86	108	46 and 58	8 to 40	(> 40) 6	60
BSP retention (%)*	7	7	9.3	12.6	7.6	0 to 10	(> 10) 4	57
Thymol turbidity (Maclagen units)	10	17	5	4	4	0 to 4	(> 4) 9	53
Prothrombin concentration (%)	10	17	48	8	43	50 to 100	(< 50) 9	53
Cephalin cholesterol flocculation	10	16	-	-	-	0 to 2 +	(> 2+) 8	50
							(> 3+) 5	31
Thymol flocculation	10	16	-	-	-	0	(> 0) 6	37
Serum pseudo-cholinesterase (Δ pH units/hour)	10	10	0.55	0.14	0.56 and 0.57	0.5	(< 0.5) 3	30
Serum albumin (Gm. %)	10	10	4.0	0.7	4.0 and 4.3	3.4 to 4.5	(< 3.4) 2	20
SGPT (S-F units)	10	10	30	38	13 and 16	5 to 35	(> 35) 2	20
Serum vitamin B_{12} ($\gamma\gamma$ Gm./ml.)	10	10	620	255	570 and 635	300 to 800	(> 800) 2	20
Alkaline phosphatase† (B-L units)	6	10	5.3	1.1	4.6 and 6.0	2.8 to 8.0	(> 8.0) 0	0

*Corrected values: Zieve and associates.[18]
†Children under 16 years of age.

Serum Vitamin B_{12}.—The greatest abnormality (2,565 $\mu\mu$g per milliliter) occurred in a patient acutely ill with fever, hepatomegaly, and jaundice (No. 13).

Urinary Coproporphyrins.—The excretion of coproporphyrins in urine may become augmented as the result of increased hemoglobin synthesis in hemolytic states, or as a consequence of liver disease[25]; both these factors may be expected to increase the coproporphyrin content of the urine in sickle-cell anemia. Urinary coproporphyrin excretion was excessive in 78 per cent of tests among the patients in Group A, and in 77 per cent of the estimations among all the subjects in the study. An inverse relationship was found between hemoglobin concentration and urinary coproporphyrin content (Fig. 1), whereas no correlation existed between urinary coproporphyrin elimination and reticulocyte counts.

The following calculation was made to determine whether the inverse relationship between hemoglobin concentration and urinary coproporphyrin excretion was statistically significant. The null hypothesis, that the true slope β of the line was equal to zero (H_o: $\beta = 0$), was subjected to the F test:

$$F = \frac{(N-2)\ (Sxy^2)}{Sxx\ Syy-Sxy^2} = 64.855$$

where x = Hb. Gm./100 ml.

y = urinary coproporphyrins γ Gm./24 hours

N = number of subjects (12)

$$Sxx = \frac{1}{N}\left\{ N\Sigma x^2 - (\Sigma x)^2 \right\} = 16.696667$$

$$Sxy = \frac{1}{N}\left\{ N\Sigma xy - (\Sigma x)\ (\Sigma y) \right\} = 4,160.416667$$

$$Syy = \frac{1}{N}\left\{ N\Sigma y^2 - (\Sigma y)^2 \right\} = 1,196,522.916$$

and the estimated line: $\hat{y} = a + bx$

where the estimated slope of the line, $b = \frac{Sxy}{Sxx} = -249.176$

and the estimated intercept, $a = \frac{1}{N}\left\{ \Sigma y - b\Sigma x \right\} = 2,360.166.$

Since F was > 21.04 the null hypothesis was rejected and it was concluded that there was a true inverse relationship between hemoglobin concentration and urinary coproporphyrin excretion with > 99.9 per cent confidence in the conclusion.

It was not clear why, in 3 patients, the excretion of urinary coproporphyrins fell within the normal range in spite of hyperactivity of their bone marrow, as shown by elevated reticulocyte counts. There was no correlation between

urinary coproporphyrin excretion and reticulocyte counts, perhaps because isolated counts do not reflect the variations in the numbers of reticulocytes which occur during a 24 hour period. It was not possible to ascertain the extent to which hepatic dysfunction contributed toward the output of urinary coproporphyrins because isomers I and III were not measured separately.

SLD, SGOT, and SGPT.—Increased activity of these enzymes probably reflects pathologic changes in several organs. Increased SLD activity has been reported in 2 patients with sickle-cell anemia[26] but the diagnosis of one of these cases is questioned because of a hemoglobin concentration of 11 Gm. per cent.

The results of the enzyme studies are shown in Table III. It is not clear what relationship exists between the serum proteins and serum enzymes but it was seen that the only two subjects (Nos. 3 and 7) in whom the proteins were normal also had normal activity of serum enzymes or a minor increase in SLD activity only (No. 7).

SERUM PROTEINS.—

The values for albumin and globulin, obtained by the biuret reaction, are given in Table II; their analysis by electrophoresis is presented in Table III.

The albumin to globulin ratio was reversed in at least 86 per cent of patients by either method of analysis. This corresponded closely with the observations of Fenichel and associates,[5] but disagreed with the report by Swaby and Branson[6] that the ratio was above normal in sickle-cell anemia.

Serum albumin was decreased in 67 per cent of subjects, an incidence greater than that reported by Green and co-workers[2] (43 per cent). The frequency of abnormalities in the globulin subfractions was as follows. The observations of Fenichel and associates[5] are in parentheses. Gamma globulin was increased in 64 per cent (80 per cent), beta globulin in 57 per cent (20 per cent) and alpha-1-globulin in 28 per cent of patients. Gamma globulin, beta globulin and alpha-1-globulin were never below normal, nor was alpha-2-globulin ever above normal. These findings disagreed with those of Bogoch and co-workers,[3] who found decreased alpha-1-globulin in 3 out of 4 patients, and increased alpha-2-globulin in one patient.

Abnormalities in the serum proteins probably reflect pathologic changes in many tissues, including the liver, and sickle-cell anemia is notoriously a disease with widespread tissue involvement.

The Exacerbation Characterized by Fever and Bone Pains.—A patient was studied while she had no symptoms (Case 10) and during an exacerbation characterized by fever and severe, generalized bone pains (Case 11). SLD increased twofold during the exacerbation, but the changes observed in BSP retention, thymol turbidity, SGPT, and SGOT indicated, if anything, that hepatic function was improved during the exacerbation.

The Exacerbation Characterized by Fever, Severe Jaundice, and Hepatomegaly (Case 13).—The acute illness of this patient was associated with evidence of marked hepatic dysfunction and was clinically indistinguishable from acute infectious hepatitis, although he had not received blood transfusions.

Bogoch and co-workers,[3] reporting a similar incident in one of their patients, thought that an illness resembling acute infectious hepatitis might be a manifestation of sickle-cell anemia. It is not unlikely that the acute hepatic disturbance was related directly to circulatory or anoxic manifestations of sickle-cell anemia within the liver.

Patients With Active Tuberculosis (Group C).—These patients did not differ significantly from the subjects in Group A with respect to liver function. Patient No. 14 had pulmonary tuberculosis; Patient No. 15 had pulmonary and vertebral tuberculosis.

Treatment of Liver Disease in Sickle-Cell Anemia.—Liver disease probably becomes worse with advancing age and may terminate in cirrhosis with hepatic failure. In many patients a history was obtained of anorexia, especially in childhood, coupled with poor economic circumstances and inferior quality of the diet. Neither Bogoch and associates[3] nor Green and co-workers[2] observed marked fatty changes in sections of liver, and there were no obvious signs of protein or vitamin deficiencies in the patients included in the present study. It is possible, however, that the onset and progress of liver disease may be averted, to some extent, by institution of proper dietary measures at an early age, for provision of adequate proteins and vitamins.

When advanced cirrhosis has supervened, it is unlikely that the most strenuous dietary measures will affect more than temporary benefit. There have been no reports concerning portacaval shunt operations in patients with sickle-cell anemia. Theoretically, this operation should allow considerable numbers of erythrocytes to bypass the liver, thereby escaping sequestration and destruction in that organ. However, in the absence of portal hypertension, the shunt, created by such an operation, will probably not remain patent.

SUMMARY

1. Hepatic function tests, measurements of the activity of SLD, SGOT, and SGPT, and analysis of the serum proteins were made on a group of 14 patients with sickle-cell anemia in whom the diagnosis was confirmed by filter paper and starch gel electrophoresis and alkali denaturation of hemoglobin.

2. Some abnormality of hepatic function was present in all patients, including those younger and relatively asymptomatic.

3. The degree of anemia was not related to the severity of hepatic dysfunction; the only correlation found, among the results of all the investigations made, was an inverse relationship between hemoglobin concentration and urinary coproporphyrin excretion.

4. Abnormalities in the activity of SLD, SGOT, and SGPT were detected in all patients, except one, whose serum proteins were also normal.

5. The observations on serum proteins confirmed the results of Fenichel and associates[5] and Green and co-workers,[2] although differences in incidence were noted. Reversal of the albumin to globulin ratio was found frequently, contrasting with the conclusions of Swaby and Branson.[6]

6. Prothrombin concentration was low in the majority of cases, a finding different from that of Walters.[24]

7. It is suggested that intensive efforts be made to provide proper nutrition at an early age for patients with sickle-cell anemia, in the hope that this may avert or delay the onset of liver disease.

The authors wish to thank Dr. G. Watson James, III for his invaluable advice; J. Atkinson, C. Hart, and Mrs. H. Weinstein, and the Department of Clinical Pathology, Medical College of Virginia, for their technical assistance; Mrs. C. A. J. Goldberg, of the Pepper Laboratory of Clinical Medicine, University of Pennsylvania, Philadelphia, Pa., for performing the starch gel electrophoresis; and Dr. M. E. Turner, for his assistance with the statistical analysis.

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ANTIHEPARIN AND HEMAGGLUTINATING ACTIVITIES OF POLYBRENE

PARVIZ LALEZARI, M.D., AND THEODORE H. SPAET, M.D.
NEW YORK, N. Y.

Hexadimethrine bromide (Polybrene), an antiheparin drug, was found to cause agglutination of human red cells and platelets. The agglutination appeared to result from neutralization of the cells' negative charge by the positively charged polymer.

The compound was also an in vitro anticoagulant when present in excess of heparin. A smaller molecular weight Polybrene was found to have neither cytoagglutinating nor significant anticoagulant effect, while being a potent heparin neutralizer. It is believed that the smaller molecular weight compound may prove to be an ideal antiheparin agent.

HEXADIMETHRINE bromide (Polybrene), a synthetic polymer of a quaternary ammonium salt (Fig. 1), has recently been used to neutralize the anticoagulant effect of heparin.[1-4] It is the purpose of this study to report the "agglutinating" effects of this compound on human red cells and platelets, a hitherto unrecognized reaction, and to describe its anticoagulant action. A low molecular weight compound of this polymer was found to have no effect on human blood cells and no significant anticoagulant activity, while being a potent heparin neutralizer; it may prove to be an ideal antiheparin drug.

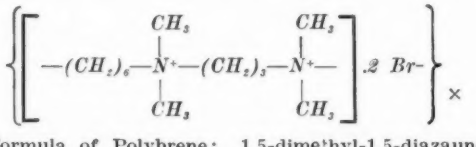

Fig. 1.—The structural formula of Polybrene: 1,5-dimethyl-1,5-diazaundecamethylene methobromide.

MATERIALS AND METHODS

Hexadimethrine bromide with average molecular weights of 6,000* and 2,000* were studied. Stock solution of each was prepared at a concentration of 10 mg. per milliliter in isotonic saline and the pH was adjusted to 7.2. Agglutination titers were determined by serial double-fold dilutions of the stock solutions in saline. These were tested against a 2 per cent suspension of thrice-washed erythrocytes in isotonic saline. Test solution and cell suspension were each added in 0.1 ml. volumes. The mixtures were allowed to stand at room temperature for 30 minutes, and the end point was determined as the

From the Blood Bank and Hematology Department, Laboratory Division, Montefiore Hospital, New York, N. Y.

Supported by the Health Research Council of the City of New York under Contract No. U-1024.

Received for publication Aug. 30, 1960.

*Supplied by Abbott Laboratories, North Chicago, Ill.

highest dilution of the polymer producing any microscopically visible hemagglutination. The final concentration of the compound at this dilution was termed the "agglutinating activity."

Additional titrations were performed with isotonic trishydoxy methyl amino methane (tris) buffer at pH 7.7 substituted for physiologic saline, and the effects of various ionic strengths of $CaCl_2$, $MgCl_2$, $BaCl_2$, $SrCl_2$, and NaCl on hemagglutinating activity were determined as follows: half-molar solutions of the above chlorides in distilled water were prepared; dilutions were made with the buffer to provide desired concentrations, and these latter were used as diluent to prepare 2 per cent suspensions of erythrocytes; serial double fold dilution of Polybrene in tris buffer were tested against these cell suspensions, as described above.

Polybrene agglutination titers were similarly performed on platelet suspensions prepared from samples of blood anticoagulated with one one-hundredth volume of 10 per cent disodiumethylenediaminetetraacetate (EDTA). Blood was centrifuged at 1,000 r.p.m. for 10 minutes, and platelet-rich plasma containing about 200,000 platelets per cubic millimeter was used. Two per cent red cell suspensions were prepared in the same plasma for comparative studies with platelets.

Heparin-neutralizing activity of the polymers was tested by clotting time determinations of heparinized blood mixed with various amounts of Polybrene. For each experiment, 100 ml. of blood obtained from healthy donors was drawn into a stoppered siliconized cylinder containing 10 mg. of heparin in 1 ml. volume. This provided 100 μg of heparin per 1 ml. blood. To each set of 3 Kahn tubes, containing from 1,000 to 50 μg of Polybrene in 0.1 ml. volume, 1 ml. of heparinized blood was added. Blood was mixed well, and then clotting times were estimated by the Lee-White method. Two sets of determinations were done on each blood specimen: (1) in which Polybrene was added at 10 minutes, and (2) in which Polybrene was added at 2 hours after blood collection, blood having been kept at room temperature.

TABLE I. SMALLEST CONCENTRATION OF POLYBRENE WHICH PRODUCED HEMAGGLUTINATION (μG/ML.)

AVERAGE MOLECULAR WEIGHT	IN BUFFER pH 7.7	IN SALINE	IN SERUM
6,000	9.7	19.5	39
2,000	5,000	*	*

*No activity with 5,000 μg/ml.

TABLE II. SMALLEST CONCENTRATIONS OF POLYBRENE WHICH PRODUCED PLATELET AGGLUTINATION (μG/ML.)

AVERAGE MOLECULAR WEIGHT	RED CELLS	PLATELETS
6,000	78	9.7
2,000	*	*

*No activity with 5,000 μg/ml.

RESULTS

Low concentrations of the 6,000 M.W. compound caused rapid clumping of human red cells and platelets, but did not affect leukocytes. The reaction was independent of blood groups; it was only slightly inhibited at 4° C., as compared to 37° C., or room temperature. A drop of 1 per cent heparin, EDTA, or sodium phytate solution added to the clumped cells caused prompt resuspension of erythrocytes, but platelet agglutination was irreversible.

The hemagglutinating activity of two polymers with molecular weights of 6,000 and 2,000, as determined in buffer, saline, and serum, is compared in Table I. Table II shows comparison of the platelet "agglutinating" activities of the two compounds, as determined in the presence of EDTA. The 6,000 M.W. compound was more effective in pH 7.7 buffer than in saline, and the presence of serum was somewhat inhibitory. Platelets proved to be an even more sensitive indicator of agglutination than erythrocytes, clumping in about one-eighth the concentration of Polybrene required for hemagglutination. The 2,000 M.W. compound had strikingly different properties. Erythrocyte agglutination was achieved only in pH 7.7 buffer and at relatively massive concentrations; no platelet agglutination was observed in the wide range of concentrations studied.

TABLE III. SMALLEST CONCENTRATIONS OF POLYBRENE WHICH PRODUCED HEMAGGLUTINATION IN PRESENCE OF VARIOUS IONS IN PROGRESSIVE CONCENTRATIONS (μG/ML.)

MICROMOLS/ML. ADDED TO ISOTONIC TRIS BUFFER	$CaCl_2$	$MgCl_2$	$BaCl_2$	$SrCl_2$	NaCl
5	9.8	9.8	9.8	9.8	9.8
10	39	9.8	19.5	19.5	9.8
15	156	39	39	39	9.8
20	*	39	39	39	9.8
25	*	39	78	78	9.8
30	*	156	156	156	9.8
40	*				9.8
50	*				9.8
125	*				78

*No activity with 5,000 μg/ml.

The effect of certain cations in varying molarity on hemagglutination is demonstrated in Table III. With sodium chloride, a marked increase in tonicity was required for agglutination inhibition. Divalent cations were inhibitory out of proportion to their increased osmolarity, Ca^{++} showing the greatest activity.

Heparin-neutralizing effects of the 6,000 and 2,000 M. W. compounds are compared in Fig. 2. Each value represents the mean of 3 determinations. It can be seen that both polymers effectively counteracted the anticoagulant effects of heparin. The 6,000 M.W. compound was active when 80 μg was used to neutralize 100 μg of heparin. However, when the Polybrene concentration exceeded twice that of the heparin, a potent anticoagulant effect was evident. The 2,000 M.W. polymer best neutralized heparin at equal concentration, but it had no significant anticoagulation effect when present at even 8 times heparin concentration. Examination of heparinized blood samples to which over 200 μg of 6,000 M.W. Polybrene was added showed marked red cell and platelet agglutination; in blood samples where heparin was neutralized with as much as 1,000 μg of 2,000 M.W. polymer, no clumping was observed. Of additional interest was the observation that the clotting time of Polybrene-neutralized blood samples was significantly shorter with a long incubation period prior to neutralization.

Rabbit red cells failed to react with 6,000 M.W. Polybrene at physiologic pH, but clumping could be produced if the cells were washed and suspended in buffered media with a pH of above 8.5.

DISCUSSION

Polybrene has been effectively used in experimental animals and humans to neutralize heparin, particularly after open heart surgery.[1-4] Untoward reactions include dyspnea, substernal pressure, lumbar pain, perspiration, flushing, and changes in blood pressure, pulse, and respiration. The role of hemagglutination in these reactions cannot be evaluated at present, but

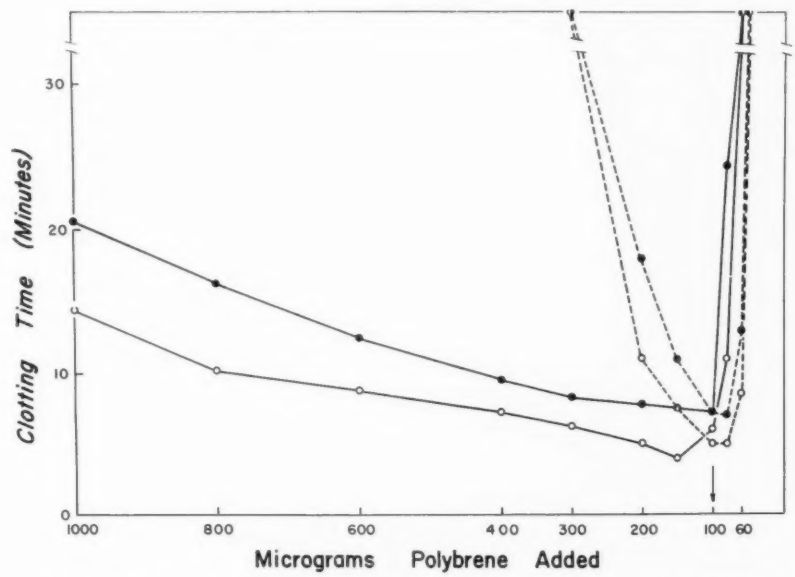

Fig. 2.—Heparin neutralization by two different polymers. The tested blood contained 100 μg heparin per millliliter.
— — — —: 6,000 molecular weight polymer.
————: 2,000 molecular weight polymer.
●: clotting time determined 10 minutes after blood was drawn.
○: clotting time determined 120 minutes after blood was drawn.

excessive thrombocytopenia observed in 30 per cent of patients[2] and in animals[3] may have been produced by in vivo platelet agglutination. Since in vitro agglutination produced by Polybrene is an almost immediate reaction, the in vivo evaluation of thrombocytopenia should be made shortly after intravenous use of the drug. In the reported cases[2-3] platelet counts were not done until 1 to 2 hours after drug administration. This delay might have been responsible for underestimation of an immediate but transitory thrombocytopenia in all cases.

The present data suggest that thromboagglutination and hemagglutination may be avoided if the smaller molecular size of Polybrene is used. Moreover, the 2,000 M.W. polymer exerted an effective antiheparin action, without being a significant anticoagulant even if used in great excess of heparin.

It has been reported that in mice the toxicity of Polybrene is increased with increase of the molecular weight.[3] It is therefore expected that use of smaller molecules of the polymer may diminish its over-all toxicity.

Agglutination caused by Polybrene may result from formation of ionic bonds between positive charges of the polymer and negative charges on the red cell[5] and platelet[6] surface. The chain of the polymer may serve as "bridges" to hold the cells together. Lack of reactivity of human leukocytes and rabbit red cells may be due to their weak negative electric charge.[5-6] It is significant that when the mean negative charge on rabbit red cells is increased by suspension in alkaline media, the cells agglutinate if mixed with Polybrene. Reversibility of hemagglutination also suggests an electrostatic bond in which red cells may be replaced by more negatively charged compounds such as heparin, EDTA, etc. Irreversibility of platelet clumping may be due to the structural specificity of these cells. The inhibitory effect of Ca^{++} ion may also be explained by competition with Polybrene for the negatively charged sites on the red cells.

Hemagglutination produced by nonimmunologic agents has long been known and is well reviewed by Jandl and Simmons.[7] Long-chain positively charged nitrogenous compounds such as polylysines have been reported to cause hemagglutination.[8] Single amino acids were not active and the activity of the polymers correlated with their chain lengths. Jandl and Simmons[7] produced hemagglutination by some metallic cations. Review of their data reveals that hemagglutination was produced only by those cations which undergo polymerization.[9] Thus, inability of cations such as Ca^{++} and Mg^{++} to produce agglutination may be explained by lack of polymerization, as is the inability of single amino acids.

SUMMARY

Commercially available hexadimethrine bromide (Polybrene), an antiheparin agent, was found to cause strong agglutination of human red cells and platelets, and it acted as a potent anticoagulant if used in excess of heparin. A smaller molecular weight compound of the same polymer was found to have neither agglutinating nor significant anticoagulant effect, while being a potent heparin neutralizer. It is believed that thrombocytopenia observed in some patients treated with commercial Polybrene might have been produced by in vivo thromboagglutination. It is suggested that a small molecular weight compound may prove to be an ideal heparin neutralizer with greatly diminished toxicity. The agglutination appears to result from neutralization of cell charge and binding of cells by the long chain polymer.

The authors wish to thank Dr. Harry Kroll for helpful suggestions and technical advice.

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ANTIBACTERIAL ACTION OF PENICILLIN IN COMBINATION WITH SERUM GLOBULINS: IN VITRO EVALUATION*

MARK BRINGHURST, M.S., AND STANLEY MARCUS, PH.D.
SALT LAKE CITY, UTAH

Gamma globulin, added to penicillin, was found to increase in vitro bioassay antibacterial action. The protein alone was devoid of action. Three strains of Staphylococcus aureus which varied in susceptibility to penicillin were used as indicators. No binding of penicillin to albumin could be demonstrated. The evidence presented suggests that more meaningful antibiotic sensitivity determinations might be carried out with either tube or disk methods which include a combination of penicillin and gamma globulin. Simple procedures for carrying out such tests are described.

EVALUATION of antibiotics by the laboratory, as a guide in clinical management, depends primarily upon two factors: the organisms isolated and the antibiotics used in the laboratory test. In many instances, however, correlation does not exist between the in vitro result and the in vivo effect.[1] One hypothesis to explain this circumstance is that current test procedures take into consideration none of the participating host defenses. On this basis, the effect was studied of the addition of serum proteins to penicillin on in vitro sensitivity of staphylococcal species.

MATERIALS AND METHODS

Organisms.—All cultures were maintained on stock culture medium† at 37° C. The following organisms were used:

1. *Staphylococcus aureus,* strain No. 1, isolated from a fatal case of septicemia at the Holy Cross Hospital, Salt Lake City, in 1957.

2. *Staphylococcus aureus,* strain No. 2, isolated in 1958 from a patient with multiple skin lesions.

3. *Staphylococcus aureus,* strain No. 3, isolated in blood culture from a patient with bacteremia in the Holy Cross Hospital in 1957.

The three strains of *S. aureus* used in this study were selected on the basis of sensitivity to potassium penicillin G. They were originally screened by means of the standard disk sensitivity method with the use of disk concentrations of 10 and 2 units; strain No. 1 was resistant to 2 and sensitive to 10 units of penicillin, while strain No. 2 was resistant

From the Department of Bacteriology, University of Utah, and Holy Cross and Latter Day Saints Hospitals, Salt Lake City.

Supported in part by Research Grant No. 2G-502 from The National Institutes of Health, Division of General Medical Sciences, Bethesda, Md.

Received for publication Sept. 23, 1960.

*Part of a thesis submitted by the first author to the Graduate School of the University of Utah in partial fulfillment of the requirements for the degree of Master of Science.

†Difco Laboratories, Detroit 1, Mich.

to both 2 and 10 units of penicillin. Strain No. 3 was sensitive to both 2 and 10 units of penicillin. In order to establish the specific sensitivity end point of the 3 different strains, broth-in-tube sensitivity tests were run. The sensitivity to penicillin of the organisms by this method was as follows: strain No. 1, 3.125, strain No. 2, 50.0 and strain No. 3, 0.18 units per milliliter of penicillin.

Assay Broth.—The broth assay medium consisted of the following ingredients per 1,000 ml. of distilled water:

Bacto beef extract	1.0 Gm.
Proteose peptone No. 3*	10.0 Gm.
Sodium chloride	75.0 Gm.
d-mannitol	10.0 Gm.
Bacto phenol red (water soluble)	0.025 Gm.

Culture and Inoculum.—The test organism was inoculated into 10 ml. of brain heart infusion (BHI) broth* and incubated at 37° C. for 18 hours. One milliliter of this culture was transferred to 9 ml. of BHI broth and incubated at 37° C. for 6 hours. Cultures prepared in this manner were diluted 1:100 with BHI broth and an inoculum of 0.05 ml. of diluted culture was pipetted into each assay tube.

Standard Antibiotic Solution.—Working solutions were prepared by dissolving 2×10^5 units of K penicillin G in 200 ml. of sterile distilled water to a final concentration of 100 units per milliliter.

Protein Additions.—Samples consisted of pooled human gamma globulin, normal and abnormal albumin (with respect to lowered gamma globulin content), and serum diluted to the desired concentration in 0.9 per cent saline (PSS).

Penicillin Tube Assay Procedure.—To tubes 10 by 100 mm. in size was added 0.5 ml. of assay medium, and the tubes were plugged with cotton and sterilized by autoclaving.

The control reference was prepared by pipetting 0.5 ml. of 100 units of penicillin into the first tube of a series of 10 tubes, each of which contained 0.5 ml. of PSS. Twofold serial dilutions were then carried out through the series of 10 tubes.

Dilution series containing varying amounts of gamma globulin, albumin, and serum were prepared in the same manner, except that the serum proteins remained constant for each assay with varying amounts of penicillin. After proper dilutions of the serum proteins and the control were made and incubated at 37° C. for 30 minutes, 0.5 ml. of each tube was added to the assay tubes and 0.04 ml. of the prepared inoculum added. The assay tubes were then incubated for 18 hours at 37° C. At the end of the incubation period, tubes of each series were observed for color change. Growth was indicated by a change in color of red to yellow; the highest dilution showing no color change was taken as the end point.

Assay Using Disk Sensitivity Method.—Trypticase soy agar† was prepared and 12 ml. amounts were distributed and sterilized in screw-cap tubes. Gamma globulin, albumin, or serum, in varying concentrations, was added to the remelted and cooled tubes just before the plates were poured. A standard inoculum, similar to the inoculum used in the test tube assay, was seeded on the plates. Disks impregnated with penicillin were placed on the medium and then incubated for 24 hours. The zone of inhibition of the growth around the disk was read in millimeters.

Electrophoretic Analysis.—Serum proteins used in this study were evaluated by zone electrophoretic technique with the use of a commercially available (Spinco) apparatus. Barbiturate buffer at pH 8.6 and 0.1 ionic strength was used in all analyses. The different serum proteins were applied in 0.01 ml. amounts as a narrow strip across a strip of filter paper moistened with electrolyte, and separation was allowed to proceed for 16 hours at 2 Ma.

*Difco Laboratories, Detroit, Mich.
†Baltimore Biological Laboratory, Md.

per cell constant current. Strips were oven dried to fix the serum proteins and were made visible by staining with phenol blue. The various bands of serum proteins were evaluated densitometrically.

RESULTS

In the presence of concentrations of albumin comparable to those in normal whole blood as well as those in blood with reduced concentrations, no measurable differences from those observed with broth were seen in penicillin sensitivity of 3 strains of *S. aureus*. The results of one of a series of experiments employing this design are shown in Table I.

TABLE I

STAPHYLOCOCCUS AUREUS AND ADDITIVE	PENICILLIN (UNITS/ML.)										
	50	25	12.5	6.3	3.1	1.6	0.78	0.39	0.18	0.09	0
Strain No. 1.—											
In broth (no additive)	–	–	–	–	+	+	+	+	+	+	+
Albumin (40 mg./ml.)	–	–	–	–	+	+	+	+	+	+	+
Albumin (20 mg./ml.)	–	–	–	–	+	+	+	+	+	+	+
Strain No. 2.—											
In broth (no additive)	–	+	+	+	+	+	+	+	+	+	+
Albumin (40 mg./ml.)	–	+	+	+	+	+	+	+	+	+	+
Albumin (20 mg./ml.)	–	+	+	+	+	+	+	+	+	+	+
Strain No. 3.—											
In broth (no additive)	–	–	–	–	–	–	–	–	+	+	+
Albumin (40 mg./ml.)	–	–	–	–	–	–	–	–	+	+	+
Albumin (20 mg./ml.)	–	–	–	–	–	–	–	–	+	+	+

Growth is represented by +, no growth by –.

To test for any antibacterial activity in the serum proteins used in this study, albumin, gamma globulin, and whole human serum were added to broth, which was then inoculated. All 3 strains of organisms grew in broth containing albumin, 40 and 20 mg. per milliliter; in broth containing gamma globulin, 2, 4, 6, 8, and 10 mg. per milliliter; and in whole normal serum, 50 and 25 per cent serum-broth. In other words, serum proteins without penicillin showed no antibacterial activity.

Waisbren[1] demonstrated, by clinical observations, that by combining gamma globulin with penicillin therapy in patients with lowered gamma globulin levels, treatment was more effective than when penicillin alone was used. To further test this observation in vitro, gamma globulin in different concentrations were added to penicillin and tube sensitivity tests were performed. The concentrations of gamma globulin employed were similar to those in normal sera and in sera showing lowered gamma globulin levels. An excess of gamma globulin was also included to determine whether above normal concentrations of gamma globulin showed a greater antibacterial effect. The results are shown in Table II.

This experiment demonstrates that as the concentration of gamma globulin increases there is greater antibacterial action of penicillin against the test organism. The antibacterial action appears to be directly proportional to the amount of gamma globulin incorporated in the tubes. When comparison is

made of the inhibitory action of penicillin alone and the antibiotic incorporated with a normal concentration of gamma globulin (8.0 mg. per milliliter) there is found approximately 35 times more activity in the case of the latter. That is, 35 times as much penicillin was required to inhibit strain No. 2 as was required to bring about this inhibition in the presence of a normal concentration of gamma globulin.

TABLE II. INCREASED ACTIVITY OF PENICILLIN IN PRESENCE OF GAMMA GLOBULIN (VARYING AMOUNTS OF GAMMA GLOBULIN WITH PENICILLIN) S. AUREUS, STRAIN NO. 1

	TUBE NUMBER										
	1	2	3	4	5	6	7	8	9	10	11
Penicillin control	–	–	–	–	+	+	+	+	+	+	+
Gamma globulin concentration (mg./ml.)											
2.0	–	–	–	–	–	–	+	+	+	+	+
4.0	–	–	–	–	–	–	+	+	+	+	+
6.0	–	–	–	–	–	–	–	–	–	+	+
8.0	–	–	–	–	–	–	–	–	–	+	+
10.0	–	–	–	–	–	–	–	–	–	–	+
Units of penicillin/ml.	50	25	12.5	6.3	3.1	1.6	.78	.39	.18	.09	0

Growth is represented by +, no growth by –.

TABLE III. INCREASED ACTIVITY OF PENICILLIN IN PRESENCE OF POOLED NORMAL SERUM (VARYING AMOUNTS OF POOLED NORMAL SERUM WITH PENICILLIN) S. AUREUS, STRAIN NO. 1

	TUBE NUMBER										
	1	2	3	4	5	6	7	8	9	10	11
Penicillin control	–	–	–	–	+	+	+	+	+	+	+
25 per cent normal serum	–	–	–	–	+	+	+	+	+	+	+
50 per cent normal serum	–	–	–	–	–	–	±	+	+	+	+
Normal serum	–	–	–	–	–	–	–	–	–	–	+
Units of penicillin	50	25	12.5	6.3	3.1	1.6	.78	.39	.18	.09	0

Growth is represented by +, no growth by –.

In similar experiments, the minimal inhibitory concentration (M.I.C.) of penicillin for strain No. 2 was 50 units per milliliter in the absence of gamma globulin; 6.3 units per milliliter with 2 and 4 mg. per milliliter of globulin; 3.1 units per milliliter with 6 mg. per milliliter of globulin; 1.6 units per milliliter with 8 and 10 mg. per milliliter of globulin. *S. aureus*, strain No. 3 was inhibited from growth in broth by 0.39 units per milliliter of penicillin. However, if as little as 2.0 mg. per milliliter of gamma globulin was added to the broth, no growth occurred in 0.09 units per milliliter of penicillin. Needless to add, no growth was observed with 0.09 units per milliliter of penicillin in the presence of 4.0, 6.0, 8.0, and 10.0 mg. per milliliter of gamma globulin.

Tube dilution tests currently in use to determine antibiotic activity in the presence of serum do not use a constant amount of serum or gamma globulin in each tube. By using the serial dilution method for evaluation of antibacterial action, one dilutes out any activity that might potentially be manifested

in the globulin component. Thus, it is possible that under these conditions the antibiotic levels determined are of the activity exerted by penicillin alone, without the benefit of the combined action with gamma globulin.

In another series of experiments, pooled normal human serum was substituted for gamma globulin. In the previous experiment it was demonstrated that the antibacterial activity of penicillin is directly proportional to the amount of gamma globulin incorporated into the medium. The serum used in the assay now described was diluted to simulate the circumstances achieved when diluting the gamma globulin. The results shown in Table III demonstrate the antibacterial potentiating activity of the serum. When diluted to 25 per cent of normal it was found that the activity of the serum is the same as that of control standard. However, as the serum approaches the normal gamma globulin range there is an increase in the inhibitory activity of the antibiotic. The M.I.C. of penicillin for strain No. 1 was 6.3 units per milliliter in broth and in 25 per cent serum, but 0.78 unit per milliliter in 50 per cent serum and less than 0.09 unit in whole serum. For strain No. 2 of the test organism the corresponding values were 50, 25, 3.1, and 0.78 units per milliliter. Strain No. 3 was not tested.

TABLE IV. ADDITION OF GAMMA GLOBULIN IN MEDIUM USING DISK METHOD FOR EVALUATION. TWO DIFFERENT STRAINS OF STAPHYLOCOCCUS AUREUS EVALUATED

	10 UNITS PENICILLIN	2 UNITS PENICILLIN
Strain No. 1.—		
Control without gamma globulin	16*	10
Gamma globulin concentration (mg./ml.)		
2.0	16	10
4.0	19	15
6.0	23	19
8.0	24	20
Strain No. 2.—		
Control without gamma globulin	10	10
Gamma globulin concentration (mg./ml.)		
2.0	10	10
4.0	12	10
6.0	16	12
8.0	20	14

*Zone of inhibition in millimeters.

At the present time, sensitivity tests are primarily carried out by the disk method. It was thought desirable, therefore, to evaluate the action of gamma globulin and serum by this method. The simplicity and rapidity of the disk method are desirable in routine laboratory procedure. As is seen in Tables IV and V, the antibacterial action, when gamma globulin and serum were added to the medium, was similar to that achieved in the tube sensitivity method. Although there is a difference in the degree of sensitivity of the two strains of *S. aureus*, the zones of inhibition are directly proportional to the amount of gamma globulin contained in the medium.

In an effort to estimate potential loss of penicillin activity in patients with decreased amounts of gamma globulin, comparative electrophoretic curves were evaluated. Table VI shows that comparable values are obtained with diluted gamma globulin and serum proteins. This test is a qualitative evaluation of the amount of gamma globulin, since only the height and the base measurements are included.

TABLE V. ADDITION OF NORMAL HUMAN SERUM IN MEDIUM USING DISK METHOD FOR EVALUATION. TWO DIFFERENT STRAINS OF STAPHYLOCOCCUS AUREUS EVALUATED

	10 UNITS PENICILLIN	2 UNITS PENICILLIN
Strain No. 1.—		
Control without serum	16*	10
25 per cent normal serum	16	10
50 per cent normal serum	17	12
Normal serum	22	17
Strain No. 2.—		
Control without serum	10	10
25 per cent normal serum	10	10
50 per cent normal serum	16	10
Normal serum	20	14

*Zone of inhibition in millimeters.

TABLE VI. PAPER ELECTROPHORETIC COMPARISON OF NORMAL HUMAN SERUM AND GAMMA GLOBULIN IN VARYING AMOUNTS

SAMPLE		PATTERN HEIGHT (MM.)	PATTERN WIDTH (MM.)
Gamma globulin (mg./ml.)	2.0	29	13
	4.0	35	17
	6.0	40	19
	8.0	50	22
Normal serum, 25%		30	14
Normal serum, 50%		37	19
Normal serum, undiluted		48	21

DISCUSSION

Differences noted between the antibiotic sensitivity of certain organisms in vitro and the results that are achieved in vivo have provided a further need for evaluation of sensitivity testing. Davis[2] has reported some of the inconsistencies observed between the standard sensitivity tests and results achieved clinically. The usual type of inconsistency observed is lack of patient response in an infectious process in which the sensitivity test has indicated an antibiotic that might be expected to yield salutary results. The opposite circumstance is not frequently observed or reported. However, Waisbren[1] found increased effectiveness of penicillin when gamma globulin was included in the therapy. The point has been stressed that present-day sensitivity tests have not included the potential action that might be elicited by incorporation of serum or gamma globulin into the medium.

Eagle, Fleischman, and Musselman[3] reported that approximately half the in vivo penicillin is bound to serum proteins and not available for antibacterial activity in this bound state. Thus, in order to correlate in vivo and in vitro

levels of penicillin, they stated that the serum levels must be 2 to 5 times greater to achieve the same action. The method used to evaluate this antibiotic activity was the standard serial dilution technique described in 1947 by Eagle and associates.[3]

Tompsett and associates[4, 5] also demonstrated this binding capacity of penicillin by albumin, but found that it varied when penicillin K or G was used. However, Jones and Finland[6] found no binding of penicillin by albumin.

The results which have been presented show no inhibitory binding of currently available penicillin by serum albumin. The discrepancies between these results and the reports of earlier investigators cannot be explained. One may speculate that with improved methods of purification of penicillin the binding capacity has been reduced.

From Eagle's[7, 8] extensive studies of the rate of destruction of the various penicillins in vivo, Davis[2] suggested that one does not obtain a true level of antibiotic concentration when correcting for low values because in these small concentrations the correction factor is very large. He also stated that under these conditions the level of antibiotic concentration is actually higher and consequently the action of antibiotic greater than is measured in an in vitro bioassay. These observations are in agreement with the work of Tillet,[9] as well as that of Jawetz[10] and Zubrod,[11] who found that pneumococci were inhibited for a considerable period of time after concentrations of penicillin in blood ceased to be measurable.

Jones and Finland[6] studied the antibacterial action of novobiocin and penicillin G and observed that in the case of the latter there was no evidence of inhibition of antibiotic effect by plasma in the concentration used. This is in complete disagreement with the observations of earlier investigators. However, Tompsett and associates[4, 5] based their findings on dialysis experiments, and Eagle[12] used a correction factor when diluting plasma for his final analysis.

The earlier investigators primarily considered the binding of chemotherapeutic agents with protein, which was inhibitory to their ultimate action, rather than the potential antibacterial action of serum proteins. MacLeod and Stone,[13] Wood and Irons,[14] and Harrison[15] have presented clinical and experimental evidence that the final destruction of a significant proportion of the invading organism depends, in most instances, upon the combined action of the antibiotic and the most natural defenses of the host.

Increased resistance of *S. aureus* to antibiotics has been consistently reported in the literature.[16-19] However, the increased resistance of *S. aureus* to antibiotics is primarily substantiated by the standard tube and disk methods of sensitivity testing. Methods currently in use do not incorporate the potential action of combined serum proteins.

Rogers,[20] in his survey of the problem of *S. aureus* infections, noted that although the organism was sensitive to penicillin by in vitro testing, in some patients the infection failed to respond to what was considered appropriate antibiotic therapy. However, Rogers[20] also found that many patients infected with

S. aureus which were penicillin resistant by in vitro tests responded to penicillin therapy. Waisbren[1] showed that addition of gamma globulins to antibiotics gave clinical results that could not be obtained when antibiotics alone were used. Fisher and Manning[21] demonstrated an increased resistance to *S. aureus* infection in mice by injecting them with human gamma globulin. Their results suggested that by increase of the amount of gamma globulin greater protection against *S. aureus* was obtained.

From results presented the hypothesis is suggested that there exists an added effect if serum proteins act in conjunction with antibiotics. The data show that by including serum proteins in penicillin sensitivity tests, whether tube or disk, increased effectiveness of the antibiotic may be demonstrated.

SUMMARY

Three strains of *Staphylococcus aureus* were evaluated for resistance to penicillin. The strains were classified as highly sensitive, moderately sensitive, and resistant to penicillin. When serum, albumin, and gamma globulin were evaluated for antibacterial activity, none was exhibited against the 3 strains of organisms.

No binding of penicillin to albumin could be demonstrated in the bioassay. The antibacterial activity of penicillin was the same when albumin was present or absent.

When gamma globulin was added to penicillin in the bioassay, increased antibacterial activity was demonstrated. The inhibitory action against the organisms was directly proportional to the amount of gamma globulin incorporated with penicillin. Gamma globulin, added in the same concentration as in normal human serum, increased the antibacterial activity to approximately 35 times that with penicillin alone. The 3 different strains of *S. aureus* appeared to behave in the same manner, although the degree of sensitivity varied.

Normal pooled serum, when substituted for gamma globulin, acted in the same manner. The antibacterial activity of serum-penicillin combinations was directly proportional to the amount of serum used in the bioassay.

Gamma globulin and normal serum were incorporated into the medium in different concentrations and standard disk sensitivity tests were performed. An antibacterial effect similar to that found with the tube bioassay was demonstrated in this disk method.

Electrophoretic patterns were determined on the different concentrations of gamma globulin and normal serum. Electrophoretically, the gamma globulin and normal serum could be equilibrated in concentrations used in the microbiologic assays.

It is suggested that the methods presented might provide a means to better evaluate antibiotic resistance of sensitivity of *Staphylococcus aureus.*

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URINARY CALCULI IN RATS ON MILK OR LACTOSE DIET

C. W. VERMEULEN, M.D., D. L. PROCTOR, M.D., AND L. SEIBUTIS, M.D.
CHICAGO, ILL.

A milk diet markedly augments stone formation in rats when a standard stimulus to stone growth, a foreign body, is placed in the urinary bladder. Urinary calcium concentration is also increased, though the intake of calcium through a milk diet was less than through a control diet of Chow. A diet containing lactose likewise increases the concentration of calcium in the urine, but the marked stone growth seen in the animals on milk diet was not duplicated in the present study. An unknown calculogenic factor in milk is suggested by these observations.

IN PREVIOUS publications[1, 2] from this laboratory various etiological factors in urolithiasis have been studied with the use of a laboratory model of the disease in rats which was produced by surgically inserting a foreign body into the urinary bladder. This technique applies the well-known clinical observation that a foreign body, when placed in the urinary tract, tends to provoke stone growth about the foreign body nidus. For experimental purposes the foreign body method has some advantage over others in that it does not require a metabolic distortion to initiate the stone-forming process. Moreover, it permits more exact determination of the amount of stone produced, since the surgically introduced foreign body cannot pass spontaneously from the urinary tract. Thus, any stone that forms upon it may be recovered and the result expressed quantitatively as stone weight. In past experiments with this method, the rats have been manipulated in a variety of ways and the resultant changes in stone growth correlated with chemical or other alterations in the urine.

The present study deals with the influence of a milk diet upon the composition of the urine and foreign body stone growth in rats. Milk and milk products are usually considered to be the most important sources of calcium in the human diet, and, consequently, patients with urolithiasis are frequently advised to restrict or eliminate milk intake so as to reduce the concentration of calcium in the urine. However, clear and unequivocal evidence of the relation between excessive milk intake and stone disease is difficult to obtain from clinical experience with patients. The variability of the disease, the uncertainty of recurrence, and the inability rigorously to control other possible etiological factors during the long period of time required for adequate study have

From the Department of Surgery, University of Chicago, Chicago, Ill.

Supported by grants from the National Institute of Arthritis and Metabolic Diseases, The National Institutes of Health, and from the Douglas Smith Foundation for Medical Research, the University of Chicago.

Received for publication Aug. 31, 1960.

all interfered with satisfactory clinical investigation of this and other etiological problems in urolithiasis. It is largely for these reasons that study with the use of a simplified laboratory model of the disease might yield worthwhile information.

METHODS AND MATERIALS

Foreign Body Insertion and Estimation of Stone Growth.—The foreign body consists of a zinc disc, 4 mm. in diameter, aseptically introduced into the bladder by cystotomy; thereupon the bladder is closed with a single suture. After an interval of 6 weeks, the bladder is re-exposed and urine aspirated for pH determination (microglass electrode) and bacterial culture on nutrient broth. The bladder is then opened and the foreign body stone removed and weighed. The actual amount of stone that had formed in the 6 week period is then calculated by subtraction of the initial weight of the foreign body. Urine culture is obtained at the final operation in order to obviate error due to intercurrent infection, which sometimes markedly alters the amount of stone produced.[3] Therefore, the observations on both stone formation and urine pH were discarded in each instance where a positive culture was obtained.

TABLE I. APPROXIMATE COMPOSITION OF DIETS

DIETARY CONSTITUENTS	CONTROL CHOW (PER CENT)	POWDERED WHOLE MILK (PER CENT)	CHOW PLUS LACTOSE OR GLUCOSE (PER CENT)
Protein	25	26	19
Carbohydrate	47	38	60
Fat	6	27	4.5
Calcium	1.3	1	1
Phosphorus	0.9	0.75	0.7
Magnesium	0.2	0.1	0.15
Calories per 100 Gm.	350	525	360
Calcium, Grams per 100 Cal.	0.37	0.19	0.28

Diets and Rat Groups.—Two different strains of young adult albino rats (weight, 150 to 200 grams) were used since it has been found that these 2 types of rat tend to produce stones of different chemical composition.[4] On our standard diet one type (Harlan males) produces stones of magnesium ammonium phosphate hexahydrate while the other type (Holtzman females) usually forms stones composed of calcium phosphate. With both types of rat 2 experiments were done in the present study. In the *first experiment* groups of 25 to 30 male and female rats were fed either our standard control diet of Purina Laboratory Chow or a diet of powdered whole milk.* The powdered whole milk used here was prepared without the usual vitamin D supplement commonly added to the commercial product. Food and water were available to the rats at all times. In the *second experiment*, again with both male and female animals, groups of 30 to 40 rats were given the following diets: (1) control Chow, (2) powdered whole milk, (3) Chow, 75 per cent—lactose, 25 per cent, and (4) Chow, 75 per cent—glucose, 25 per cent. Stone growth with Diets 1 and 2 of the *second experiment* did not differ from the corresponding groups in the first study, so the 2 experiments have been combined in the description of the results obtained. Some pertinent figures on the composition of these diets are given in Table I. It is important to note that the concentration of calcium in the control diet (1.3 per cent) is actually greater than that in the powdered milk diet (1.0 per cent). Moreover, since the caloric value of powdered milk is higher than that of the control Chow, the total food intake of rats on a milk diet would be considerably less than that of rats on the control diet, if equal caloric intake were assumed for both diets. If the caloric intake was, in fact, equal in rats on either of the 2 diets, the calculated intake of calcium through the milk diet would be approximately half that obtained

*The supply of powdered milk used in these experiments was furnished by the Dean Milk Company, Franklin Park, Ill.

through the Laboratory Chow. It proved to be impractical to determine total food intake with exactness, but some indication of food consumption is available in average weight gains of the rats during the 6 week experimental period. For male rats on control Chow, milk, lactose, or glucose diets, the average weight gains were 52, 55, 59, and 87 grams, respectively. Corresponding figures for the groups of female rats were 32, 44, 31, and 54 grams. Thus, except for the rats on the glucose diet, the animals grew on the different diets at approximately the same rate during the experimental period, the males more rapidly than the females.

Urine and Stone Analysis.—Quantitative 24 hour urine collections were obtained in the third and fifth weeks of the second experiment. In each of the 8 groups of rats a total of 30 to 50 urine collections were made, each consisting of a 24 hour specimen from 2 rats. To prevent precipitation of insoluble salts during collection, 1 ml. of 4N HCl was added to the urine collector beneath the metabolism cage, corrections being subsequently made for this 1 ml. of additional volume. Each urine specimen was analyzed for Ca, Mg, and P by methods previously noted.[1, 2] No differences were observed between the 3 and 5 week values and they were therefore averaged together for each of the dietary groups. In the *second experiment* the type of stone formed in each group was determined by analysis of 15 randomly selected stones for Ca, Mg, and P. However, this analysis was omitted in 2 groups because stones of sufficient size were not available.

TABLE II. SUMMARY OF STONE FORMATION

TYPE OF ANIMAL AND DIET	NUMBER UNINFECTED RATS*	AVERAGE WEIGHT OF STONE ON Zn DISC (MG.)	CALCIUM CONTENT OF STONE (PER CENT)	NUMBER RATS WITH STONES IN UPPER URINARY TRACT
Harlan male rats.—				
Chow diet	46 (6)	63	0	0
Powdered whole milk	50 (14)	111	25.7	3
Chow—lactose	26 (3)	53	8.8	0
Chow—glucose	22 (2)	88	0	0
Holtzman female rats.—				
Chow diet	46 (5)	13		0
Powdered whole milk	27 (23)	91	24.9	23
Chow—lactose	28 (8)	52	24.1	3
Chow—glucose	31 (2)	12		0

Statistical "p" values—weight of stones on Zn discs.—

	MALE RATS	FEMALE RATS
Milk vs. control	$<.001$	$<.001$
Lactose vs. control	$>.05$	$<.001$
Glucose vs. control	$>.05$	
Milk vs. lactose	$<.001$	$<.001$

*Figures in parentheses indicate additional rats which survived to end of experiment but were discarded from the results because positive urine cultures were obtained.

RESULTS

Growth of Stones on Foreign Bodies, Control Vs. Milk Diet (Table II).—Of the Harlan male rats 46 control and 50 milk-fed animals survived the 6 week experimental period and were uninfected at the time of sacrifice. The average weight of stone formed on the foreign body in the control animals was 63 mg., while the group of rats on the milk diet had almost twice that amount, an average of 111 mg. The chemical nature of the stones was also changed; in the control rats the stone salt consisted of magnesium ammonium phosphate, while in the rats on the milk diet it became a calcium phosphate with an average

composition of 25.7 per cent calcium and 17 per cent phosphorus. Of the Holtzman female rats, the 46 control animals produced an average foreign body stone of only 13 mg., while the 27 rats fed a milk diet yielded an average of 91 mg., or almost a seven fold increase. The stones formed in the control group were too small for satisfactory analysis; those formed in rats on the milk diet were of the calcium type, containing 24.9 per cent calcium and 17.6 per cent phosphorus.

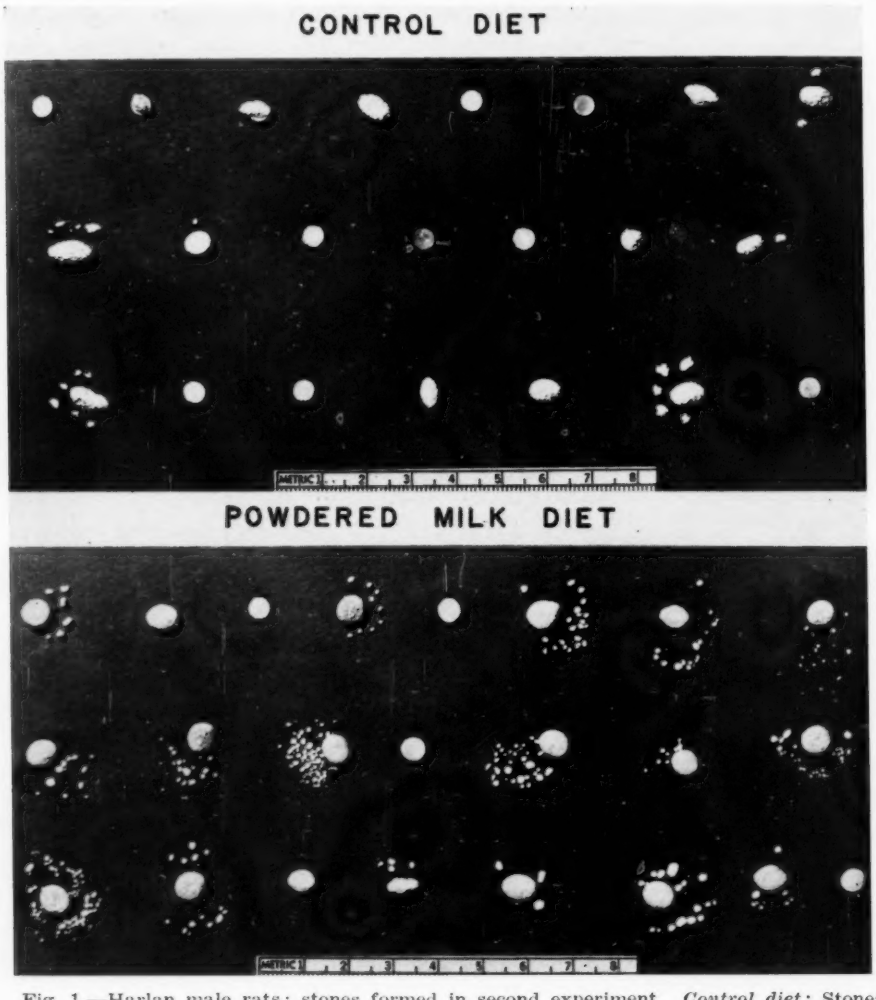

Fig. 1.—Harlan male rats; stones formed in second experiment. *Control diet:* Stones of varying size have developed about the Zn nuclei. (A foreign body upon which stone formation did not occur is seen in center of photo.) The "daughter" stones found in these rats are shown clustered about the primary foreign body stones. *Milk diet:* Foreign body stones are larger and development of daughter stones much more marked. In one rat of this group a small kidney stone (not shown) was also found.

(In previous experiments with Holtzman female rats, the control diet has yielded somewhat larger stones than in the present study; when analyzed they proved to be of the calcium variety, with a composition similar to that found here in rats on the milk diet.) The incidence of infection was considerably increased in rats on the milk diet. Of both male and female animals, 12 per cent

of the control rats were infected, while of the rats on milk diet, 22 per cent of the male and 46 per cent of the female rats yielded a positive culture and hence were discarded from the experiment. Presumably, this predisposition to intercurrent infection is a reflection of the greater chance of temporary obstruction due to increased formation of accessory stones in the milk-fed rats. It may be noted, however, that the animals receiving milk gave no outward evidence of illness and that the gain in body weight during the 6 week period of study did not significantly differ in rats on the 2 diets.

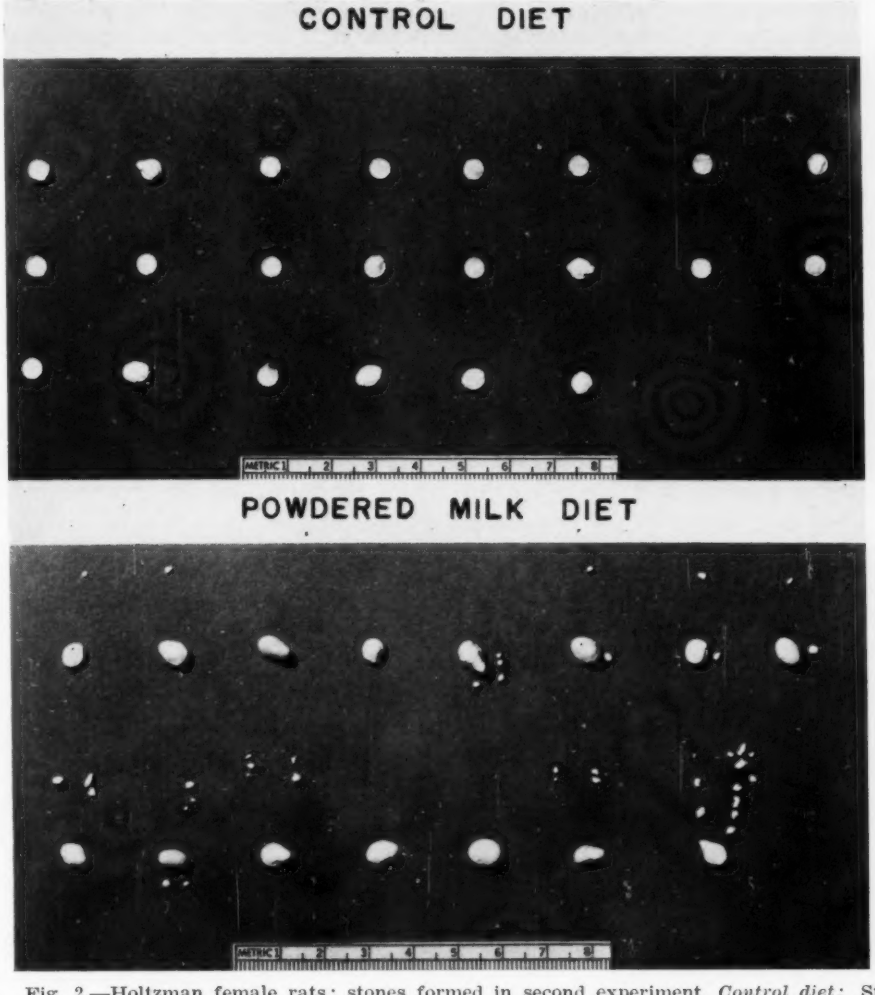

Fig. 2.—Holtzman female rats; stones formed in second experiment. *Control diet:* Stone formation is minimal. *Milk diet:* Large foreign body stones have formed. Accessory bladder stones are shown to the side or below the corresponding foreign body stones, while the kidney and ureteral stones that formed in these rats are seen above the bladder stone in each instance.

Accessory Stones in Male Rats on Milk Diet.—In previous studies of stone growth on foreign bodies, the development of accessory "daughter" calculi has been observed frequently in male rats when growth of the primary foreign body

stone has been particularly active. However, in the past we have not attempted to quantitate accessory stone development, since we believed it would be too inaccurate; many such stones would be lost during the course of the experiment by passing through the urethra when they were still of small size. Despite this objection, an effort was made in the present study to recover and weigh all accessory stones because their development on milk diet was strikingly exaggerated. Daughter calculi were found in 86 per cent of the group of rats on the milk diet as compared with 20 per cent found in the control rats. The average weight of accessory stones in the milk-fed rats was 35 mg. per rat as compared with an average of only 4 mg. for the control animals. Fig. 1 illustrates these differences by comparison of the bladder contents of the male rats on control and milk diets in the *second experiment*. Development of stones in the kidney was also found in 3 of the 50 male rats receiving milk. Such stone growth in the upper urinary tract has not been observed in many hundreds of rats on control Chow diet in our past experiments.

Accessory Stones in Female Rats on Milk Diet.—In female animals daughter calculi in the bladder are found only rarely, doubtless because the shorter, more patulous urethra permits easy passage of these small stones. However, on the milk diet, 27 per cent of the rats were found with accessory bladder stones at the time of sacrifice, though they were few in number in any individual rat. The female rats given milk differed from the males in that stones were found in the upper urinary tract far more frequently. In male rats the incidence was only 6 per cent, while stones were found in the kidney or ureter in 85 per cent of the females. Moderately advanced hydronephrosis or hydroureter was often present as a result of blockage by the stone. Stones in the upper urinary tract were not seen in any of the female rats on control diet. Fig. 2 is an attempt to depict this difference by showing the results in the female rats from the *second experiment*.

Effect of Dietary Lactose on Stone Growth.—As summarized later in this paper, several investigators have shown that a diet containing large amounts of lactose leads to increased absorption and utilization of calcium. Therefore in the *second experiment* groups of male and female rats were given a diet composed of Purina Laboratory Chow fortified with 25 per cent lactose to determine whether the calculogenic effect of milk could be reproduced by a non-milk diet high in lactose. For additional control, other groups were given a similar diet containing 25 per cent glucose instead of lactose. (The sugar level of 25 per cent was selected to give the same daily lactose intake as with powdered milk, which has a caloric value higher than that of the control Chow.) As seen in Table II, lactose did not yield complete reproduction of the type and severity of stone growth found in rats on the milk diet. In the male rats the foreign body stones were less than half the size obtained from rats on the milk diet and formation of daughter calculi in the bladder was no more frequent or severe than in the control rats. Similarly, lactose given to female animals resulted in smaller foreign body stones, and accessory daughter calculi in the bladder were absent. The lactose diet was capable, however, of inducing stone formation in the upper

urinary tract in 11 per cent of the rats, but not to the extent found with milk, where the incidence was 85 per cent. Thus a Chow diet modified to contain 25 per cent lactose resulted in effects similar in type to those found with the milk diet, but not to the same degree or severity. The diet fortified with glucose yielded results essentially no different from the control Chow diet.

TABLE III. AVERAGE VALUES ON EXAMINATION OF URINE

TYPE OF ANIMAL AND DIET	URINE pH AT SACRIFICE	ANALYSES OF 24 HOUR URINE COLLECTIONS*			
		VOLUME (ML./RAT/ DAY)	CALCIUM (MG. PER CENT)	PHOSPHORUS (MG. PER CENT)	MAGNESIUM (MG. PER CENT)
Harlan male rats.—					
Chow diet	6.5	9.2	12.2	185	46
Powdered whole milk	6.2	12.4	24.7	187	33
Chow—lactose	6.0	5.6	49.0	210	63
Chow—glucose	6.8	6.5	10.8	220	53
Holtzman female rats.—					
Chow diet	6.7	7.2	26.5	190	51
Powdered whole milk	5.8	12.7	71.0	155	32
Chow—lactose	6.2	5.8	84.1	150	59
Chow—glucose	6.4	5.4	22.0	180	52

*Each figure represents average values from minimum of 30 analyses on 24 hour collections from 2 rats.

Analyses of the Urine (Table III).—The data on mineral excretion are expressed in terms of concentration since stone formation is presumably analogous to precipitation and therefore dependent on degree of saturation rather than on total amounts excreted per day. The most striking urinary change in rats on the milk diet was the increase in concentration of calcium. The average levels were 2 and 3 times the respective control values in the male and female rats. Phosphorus concentration was not significantly altered, while magnesium was moderately reduced, a finding that is not surprising, since milk contains less magnesium than the control diet. Both the volume and acidity of the urine increased, but these changes would tend to inhibit rather than favor stone growth. Thus one might conclude that stone augmentation in rats on the milk diet was due entirely to the increased concentration of calcium in the urine. However, the results with the lactose-fed groups suggest that such a simple explanation is not completely adequate, for though the levels of urine calcium received through lactose were as high or higher than those obtained from milk, lactose did not provoke the striking stone growth seen in rats on the milk diet.

DISCUSSION

In 1923 Dragstedt and Peacock[5] noted that a diet very high in lactose delayed or prevented tetany in parathyroidectomized dogs. This was soon confirmed by Inouye,[6] who also observed that administration of parenteral lactose was ineffective. Bergeim[7] then established that a diet of 25 per cent lactose, when given to rats, resulted in marked increase in calcium absorption, due, he believed, to an increase in intestinal acidity. In 1935 Dodge,[8] also working with rats, reported that a casein-lactose diet frequently produced cataracts, and noted in the same paper that vesical calculi occurred in some of

his experimental animals. Subsequently many investigators have studied cataract formation as well as calcium utilization in laboratory animals on diets containing sugars such as lactose, galactose, xylose, or arabinose.[9-10] Apart from their interesting cataractogenic properties, it is clearly established that these sugars, when present in the diet in large amounts, markedly increase calcium absorption in rats and other animals, though the mechanism of action is as yet uncertain. Since milk contains large amounts of lactose, calcium absorption in animals on a milk diet should be greater than would be expected from its calcium content alone. Several authors[18-21] have observed that this is so, at least for the rat. In some studies[22, 23] urinary calculi and calcifications have been found when the animals were fed milk for extended periods of time.

In man the evidence for increased calcium absorption and utilization from milk or lactose additions to the diet is not entirely clear. Steggerda and Mitchell[24] and Patton[25] report that adults utilize calcium equally well whether it be added to the basal diet as milk, calcium gluconate, or calcium carbonate. On the other hand, Mills and associates[26] report a 33 per cent increase in calcium retention in young boys when lactose was added to a basal diet, the calcium being in the form of $CaHPO_4$. Thus there would seem to be some discrepancy between the effects found in man and the rat. However, it should be noted that milk (or its lactose equivalent) constituted a much higher proportion of the total diet in the animal experiments than in the human studies just mentioned.

Of course, in addition to lactose, many other factors are known to influence calcium absorption and utilization in rats. Among these are vitamin D, the dietary fat content,[27] and the previous level of calcium intake prior to the test period. An influence less commonly appreciated is the ratio of calcium to phosphorus in the diet. This laboratory[1, 2] has reported that the addition of excess phosphate to the diet of rats is capable of reduction of the urinary calcium concentration to such an extent that formation of calcium phosphate stones is prevented, a seeming paradox since the formation of stones can actually be prevented by the addition to the diet of one of the ionic components of the stone. The converse, suppression of urinary phosphate by the addition of excess calcium to the diet, likewise inhibits calculus formation. Dietary protein also is reported to affect calcium absorption from the gut. McCance and associates[28] showed that absorption and retention of calcium in human subjects on the high protein diet was 3 times that found in those on a low protein intake. Wasserman and co-workers,[29] with the use of radioactive techniques, assayed the effect of 18 amino acids on calcium absorption in the rat; they concluded that L-lysine, L-arginine, and tryptophan favored absorption, though not to the extent of lactose, which was studied simultaneously. An interesting observation, perhaps especially pertinent to the peculiar absorbability of calcium from milk, has been introduced by Mellander[31, 32] and Reeves and Latour.[33] They have shown that phosphopolypeptides formed from casein are potent complexing agents for calcium and that they enhance the solubility of calcium phosphate. These authors suggest that casein polypeptides may play a significant role in the absorption and transport of calcium.

The findings obtained in the present study are in general agreement with some of the observations in rats that have just been reviewed. By combination of a milk diet with the local stimulus of a foreign body in the bladder, stones are produced in a shorter time and in a way that permitted estimation of stone growth in a more quantitative manner. Though the total intake of calcium in rats on the milk diet was presumably much less than in those on the control Chow, stone formation was much more pronounced in rats fed milk. The results obtained with a lactose diet are interpreted, therefore, as indication that the milk effect on stone growth is not entirely due to its lactose content. Conceivably, casein might be the additional factor in milk if, as Mellander suggests, some of the calcium chelate with casein phosphopolypeptide can be absorbed from the gut as such. If so, one might suppose that a portion would be excreted unchanged in the urine and there contribute to excessive stone formation in some unknown way. Such a speculative notion is admittedly contrary to the solubilizing property of the polypeptide but somewhat attractive when it is remembered that urinary calculi invariably contain a matrix component composed of protein, the origin of which is as yet not understood.

Though extrapolation from one species to another may not be undertaken lightly, the enhancement of stone growth in rats on a milk diet suggests the possible significance of a high milk intake in causation or aggravation of human urolithiasis. In clinical practice milk restriction is, in fact, generally advised for patients who recurrently form stones, but the reason ordinarily given is the content of calcium in the milk. A further and perhaps more important reason for milk restriction is to be found in its content of lactose which, by facilitation of calcium absorption, leads to urinary concentrations much higher than would be expected from its calcium content alone. Moreover, milk appears to promote stone formation, at least in rats, by still other mechanisms, which are as yet unknown. If confirmed, an explanation of this special action could provide fundamental information on the pathogenesis of urolithiasis, a disease which, unfortunately, is still not completely understood. The experiments also raise questions regarding the causation of the milk-alkali syndrome and the dangers of the ingestion of large amounts of milk and alkalis by the patient with a peptic ulcer. Further work on these aspects is in progress.

SUMMARY

The effect of a powdered milk diet on urinary calcium and calculus formation was studied in rats by means of a surgically inserted foreign body into the urinary bladder as the local stimulus to stone growth. On the milk diet the rats received less calcium than they did on the control Chow, yet the average foreign body stone weight was 2 and 7 times larger for male and female rats, respectively. Milk also provoked the development of numerous accessory stones. In males these were located primarily in the bladder, while in females many were found in the upper urinary tract.

A milk diet increased the urinary calcium concentration to 2 to 3 times the control values, apparently because the lactose content of milk facilitates calcium absorption. Lactose, when added to the control diet, also produced

urinary calcium concentrations as high as those seen in rats fed milk, but stone formation was much less marked. It is suggested that the calculogenic property of milk is due in part to the excessive urinary concentrations of calcium, and in part to other unknown factors. Some possible clinical implications are mentioned.

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ELECTROPHORETIC STUDIES OF LEUKOCYTE ANTIBODIES

I. Evaluation of Experimentally Produced Antibodies

Gloria D. Eng, M.D.,* and Sanford L. Leikin, M.D.**
Washington, D. C.

Numerous techniques have been developed for the detection of leukocyte antibodies. Leukoagglutinin methods have been the most widely employed. However, difficulty has been encountered in the interpretation of leukoagglutinin tests because of false positive clumping of leukocytes in control sera and the absence of positive reactions when they are expected. It has been shown that when small particles are coated with various protein antibodies a change in the electrophoretic velocity is produced. This principle was applied in the development of a new method for determination of the presence of leukocyte antibody.

Anti-guinea pig leukocyte antibody was produced in rabbits with the aid of Freund's adjuvant. Guinea pig leukocytes were incubated with this antibody and then electrophorized in the microelectrophoresis cell of Ponder. The electrophoretic mobility of these cells was compared with that of similar leukocytes treated with saline and nonimmune serum. A definite decrease in electrophoretic velocity was noted in the antibody-treated leukocytes.

This technique offers a rapid and accurate method for the detection of leukocyte antibodies.

COGENT evidence for the existence of leukocyte antibodies has been presented by investigators in Europe and in the United States.[1-11] These investigators have described the presence of such antibodies in patients suffering from agranulocytosis caused by specific drug intolerance, acquired hemolytic anemia, aleukemic leukemia, thrombocytopenia purpura, disseminated lupus erythematosus, and nonhemolytic transfusion reaction. They have employed multiple techniques in the demonstration of these antibodies, such as inhibition of polymorphonuclear leukocyte phagocytosis, decrease in white cell motility, quantitative determination of lysis of exposed leukocytes, and administration of the Coombs consumption test. The most favored method, to date, is the leukoagglutinin technique, which consists of the clumping of white cells upon their exposure to the antibody. In spite of its apparent simplicity, this method produces erratic results. The primary problem lies in the absence of a satisfactory method for the separation of viable and uncontaminated leukocytes.

From the Research Foundation of the Children's Hospital of the District of Columbia, Washington, D. C.

Supported in part by the Alexander and Margaret Stewart Fund.
Received for publication May 25, 1960.

*Public Health Service Postdoctorate Research Fellow.
**Hematologist, Research Foundation of the Children's Hospital of the District of Columbia, Washington, D. C.

Other difficulties include the exaggeration or inhibition of agglutination reactions by anticoagulants and fibrinogen, and false positive reactions which actually represent erythrophagocytic activity and "leukergy." The search for an improved technique for the detection of leukocyte antibody resulted in the present study.

The electrophoretic mobility of erythrocytes, erythrocyte "ghosts," bacteria, and spermatozoa has been observed by Abramson, Moyer, Northrop, and Ponder.[12-16] Leukocyte mobility has been studied in France by Robineaux and Bazin[17-19] with utilization of Choucron's electrophoretic cell. In 1945, Coombs[20] demonstrated slowing of the electrophoretic mobility of Rh-positive erythrocytes following incubation with sera containing complete and incomplete Rh antibody. Ponder[21] has observed slowing of platelet velocity by coating these particles with dextrans high in molecular weight. These changes in velocity were presumably due to a change in surface charge. Alteration of the surface charge of leukocytes by absorption of antileukocyte antibody should similarly affect their migration characteristics in an electrical field. The following study is a test of this hypothesis.

MATERIALS AND METHODS

The preliminary phase of the study involved establishment of the technique of microelectrophoresis of leukocytes. The vertical electrophoresis cell of Ponder was chosen as the most suitable instrument.* Phase contrast microscopy was used to enable direct observation of the migration of the cells. The objective was focused to a depth of 0.21 mm. of the cell chamber, the upper "stationary" level, to obviate endosmotic flow. The cells were suspended in Michaelis Universal buffer at pH 7.4. Current was applied at 225 v. and 15 Ma. dilutional factors, specific conductance, viscosity of the suspension, and temperature were held constant. The cells were observed for a 5 second interval and the distance traveled measured by a calibrated micrometer disc. Calculations were made in relation to an erythrocyte standard. Under controlled operational conditions, a normal human erythrocyte traveled 5 spaces of the calibrated micrometer disc in 5 seconds. Its velocity was determined to be 1 μ per second per volt per centimeter. The electrophoretic velocity of both normal human and guinea pig leukocytes was determined to be 0.7 μ per second per volt per centimeter.

The next phase of the study involved the electrophoretic observation of guinea pig leukocytes with and without exposure to anti-guinea pig leukocyte antibody. An antigen-adjuvant mixture was prepared in accordance with the method of Moeschlin.[2] Guinea pigs were injected intraperitoneally with 20 ml. of sterile 5 per cent glucose in beef broth. Twenty-four hours later, 50 ml. of 0.85 per cent sodium chloride was injected into the peritoneal cavity, and 4 hours later the cavity was carefully aspirated. The solution obtained was centrifuged slowly. The supernatant was discarded, and the button of packed cells was washed 3 times with 0.85 per cent sodium chloride. The white cells were mixed with an equal volume of Freund's adjuvant. The antigen-adjuvant mixture was then stored in a frozen state.

The antileukocyte antibody was produced in rabbits by subcutaneous injections of 1 ml. of the freshly thawed antigen-adjuvant mixture at weekly intervals for 6 to 7 weeks. Three weeks after the last injection, 1 ml. of a saline suspension of freshly prepared leukocytes was injected intravenously. The presence of adequate antileukocyte antibody in the sera of these rabbits was determined by the leukoagglutinin test. This was further substantiated by the intracardiac injection of guinea pigs with the immune sera which produced

*We are indebted to Dr. Eric Ponder for his invaluable advice and encouragement in the development of this technique.

a definite leukopenia. When antibodies reached satisfactory levels, blood was obtained under sterile precautions from the ear veins of the rabbits. The serum was collected and heated to 56° C. for 20 minutes to inactivate complement. Three or 4 absorptions by equal quantities of washed guinea pig red cells were necessary for removal of erythrocyte antibodies. The serum was then sealed in sterile vials and stored at 20° C.

White cells prepared for agglutination reactions and electrophoretic studies had to be intact. This was accomplished by injecting guinea pigs intraperitoneally with 20 ml. of 3.5 per cent solution of polyvinylpyrrolidone (Subtosan). Twelve to 18 hours later an additional 20 ml. of the same solution was injected. Three hours after the last injection the peritoneal exudate was aspirated. At this point the actual testing for the presence of antibodies was initiated. First, the leukoagglutinin test was performed by the method of Dausset and associates.[4] This was done by making serial dilutions of the rabbit sera containing anti-guinea pig leukocyte antibody and adding these to guinea pig leukocytes. After gentle agitation, the tubes were placed in a water bath at 37° C. for 1 hour. A small amount of 1 per cent acetic acid was added to each tube to lyse any contaminating erythrocytes. A drop from each tube was then placed on a glass slide and examined under the microscope at low magnification; the leukoagglutinin reaction was then graded. Finally, the electrophoresis was performed by suspending a portion of each of the above tubes in 10 ml. of Michaelis universal buffer adjusted to pH 7.4, in the vertical electrophoretic apparatus previously described. The electrophoretic mobility of leukocytes from control tubes incubated with physiologic saline and several nonimmune rabbit sera was also determined at the same time.

TABLE I. SUMMARY OF THE ELECTROPHORETIC MOBILITY OF GUINEA PIG LEUKOCYTES FOLLOWING INCUBATION WITH RABBIT ANTILEUKOCYTIC AND CONTROL SERA

TEST MEDIUM	MEAN VELOCITIES (μ/SEC./VOLT/CM.)	RANGE OF VELOCITIES (μ/SEC./VOLT/CM.)	STANDARD DEVIATION	COEFFICIENT OF VARIATION (PER CENT)
Antiserum 1	0.41	0.4 to 0.5	0.027	6.7
Antiserum 2	0.46	0.4 to 0.5	0.050	2.2
Nonimmune sera (controls) 1	0.68	0.6 to 0.7	0.020	3.0
2	0.66	0.6 to 0.7	0.051	7.7
Saline (controls) 1	0.71	0.7 to 0.8	0.032	4.5
2	0.64	0.6 to 0.7	0.050	7.8

RESULTS

The leukocyte electrophoresis and leukoagglutinin test were performed on 3 separate occasions on each serum specimen with nonimmune and saline controls. Each time 2 observers evaluated the samples without knowledge of the contents of the tubes. The final results of the leukocyte electrophoresis test was the mean of 12 separate readings taken on each specimen.

Two different antileukocytic sera with titer of 1:16 and 1:32 against guinea pig leukocytes determined by the agglutinin technique were used. Both observers noted upon electrophoresis a definite difference between the rate of migration of the antibody-treated cells and their controls. The leukocytes incubated with the 2 different immune antileukocytic sera had velocities in the range of 0.4 to 0.5 μ per second per volt per centimeter. The cells exposed to 2 nonimmune rabbit sera had velocities of 0.6 to 0.8 μ per second per volt per centimeter and those exposed to the saline, 0.6 to 0.8 μ per second per volt per

centimeter. A tabulation of the electrophoretic velocities of guinea pig leukocytes after incubation with the immune sera compared to similar cells incubated with a group of nonimmune sera and saline are shown for one experiment in Table I.

A comparative analysis of the results of dilutions of the sera by the agglutinin and electrophoretic technique proved inconclusive. In some experiments the agglutinin method was positive one tube dilution beyond the electrophoretic technique. In other observations the latter technique was more sensitive.

Certain investigators[22] have observed platelet slowing when these particles were suspended in serum and have attributed this delay to coating with globulins. In this study the leukocytes exposed to control nonimmune serum did not migrate significantly slower than cells exposed to physiologic saline.

DISCUSSION

The electrophoretic mobility of a cell in an electrical field is governed principally by the distribution of the electrical charge on its surface. The electrical properties of the cell membrane and the surrounding liquid medium constitute a solid-liquid interface which has a comparatively large difference in electric potential. Because of this gradient at the interface, the cell migrates toward or away from an electrical pole in response to an external source of current. When a cell surface is coated with protein material, a change in its electrical potential occurs which affects its mobility in an electric field.

Abramson[12, 13, 22] utilized this physical principle in the study of numerous materials. He observed inert quartz particles and oil droplets and found that they assumed the mobility of the protein in which they were bathed. Erythrocytes, however, generally preserved their surface integrity but did unite with heterologous serum proteins in special sensitization reactions. He postulated the "key spots" theory in this regard, i.e., that certain critical sites break down on the cell membrane, permitting protein adsorption with a concomitant change in electrophoretic mobility. Platelets have been shown by means of a similar technique to be at least partially coated by protein solutions.

In this study, the electrophoretic method has satisfactorily detected the presence of artificially produced antibody. This technique exhibits distinct advantages over others. Considerable reliance has been placed upon the agglutination technique in the past. However, false positive and negative reactions due to various phenomena have produced erratic results. The method described here, which is based on a change in the surface charge of the white cell, circumvents these difficulties. Furthermore, a prozone phenomenon is eliminated. The method is rapid and facility in manipulation of the electrophoretic apparatus is easily acquired. The use of high magnification in the optical system enhances sensitivity and permits identification of the specific cell types.

The precision and accuracy of the instrument are reflected in the coefficient of variation (range 2.2 to 7.8 per cent) obtained in our reading. As this technique offers a rapid, accurate, and sensitive method for the detection of

leukocyte antibodies, its application to patients with leukopenia, agranulocytosis, and nonhemolytic transfusion reactions seems worthy of investigation. Such a study of clinical states will be detailed in a subsequent publication.

SUMMARY

An electrophoretic technique for the detection of leukocyte antibody is described. Precise and consistent results were found in the study of guinea pig leukocytes upon exposure to anti–guinea pig leukocyte sera as compared to appropriate control elements.

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ELECTROPHORETIC STUDIES OF LEUKOCYTE ANTIBODIES

II. Study of Clinical States

Gloria D. Eng, M.D.,* and Sanford L. Leikin, M.D.**
Washington, D. C.

A microelectrophoretic method for the detection of leukocyte antibody has been shown to give satisfactory results using rabbit anti–guinea pig leukocyte sera.

An evaluation of the technique, therefore, was undertaken in relation to two clinical problems. Its applicability was tested in the detection of leukocyte antibody in various clinical states associated with such antibodies. This technique gave reproducible results and correlated well with the leukoagglutinin method.

Second, sera containing antierythrocyte antibody were incubated with leukocytes from donors with the heterologous blood groups. Antibody could not be detected on the surface of such leukocytes by microelectrophoretic means.

A METHOD which circumvents the use of the agglutination technique has been described for the detection of leukocyte antibody.[1] This method consists of the determination of the electrophoretic mobility of white cells following exposure to a test serum. In a previous paper[1] it was shown that a retardation of the electrophoretic mobility of guinea pig white cells occurred after incubation with a rabbit anti–guinea pig leukocyte serum. This paper describes our observations with the use of this method in several clinical problems.

MATERIAL AND METHODS

Sera from patients who had hematologic disorders and who were suspected of having a leukocyte antibody were obtained from our own hospital and from other investigators in this field.† These sera were primarily from subjects who had granulocytopenia, pancytopenia, or leukemia, or who had exhibited nonhemolytic transfusion reactions.

Sera were also collected from several different blood donors, listed as follows: (1) A type O Rh-positive donor with an anti-A saline titer of 1:4,096 and immune titer of 1:20,480; (2) a type O Rh-positive donor with an anti-B immune titer of 1:24,500; and (3) a type O Rh-negative individual with a saline anti-D titer of 1:16, albumin titer of 1:256, and an indirect Coombs titer of 1:2,048. All sera were stored in sterile containers at 20° C. and inactivated at 56° C. prior to use. Fresh autologous serum was also used in several studies, as cited below.

From the Research Foundation of the Children's Hospital of the District of Columbia, Washington, D. C.

Supported in part by the Alexander and Margaret Stewart Fund.

Received for publication May 25, 1960.

*Public Health Service Postdoctorate Research Fellow.

**Hematologist, Research Foundation of the Children's Hospital of the District of Columbia, Washington, D. C.

†Specimens were submitted by Drs. Thomas W. Brittingham, John F. Fortran, John J. Butler, James L. Tullis, and Howard A. Pearson.

The leukocytes were obtained from blood donors with A and B Rh-positive, O Rh-positive, and O Rh-negative erythrocyte typing. The blood was placed in siliconized centrifuge tubes which contained 5 per cent disodiumethylenediaminetetraacetate and was allowed to sediment for 1 hour. The leukocyte-rich plasma was removed and centrifuged at 800 r. p. m. for 30 minutes at 5° C. The white cell button was then resuspended with approximate diluent so that the cell concentration was at least 10,000 per cubic millimeter.

The slide agglutination test was done according to the method of Dausset and associates.[4] The leukocyte electrophoresis was accomplished in the vertical apparatus according to the method described previously.[1] The final result for any particular serum was the mean of a minimum of 5 to 10 readings. A decrease in the electrophoretic velocity signified the presence of antibody on the white cell surface.

TABLE I. RESULTS OF LEUKOCYTE ANTIBODY STUDIES IN 20 PATIENTS

PATIENT	DIAGNOSIS	LEUKOAGGLUTININ	RANGE OF ELECTROPHORETIC VELOCITY (μ/SEC./VOLT/CM.)
0	Normal controls (30 different sera)	0	0.6 to 0.8
1	Thalassemia major N.H.T.R.*	2+	0.4 to 0.5
2	Placenta previa with postpartum cortical necrosis and febrile reaction following artificial kidney	+	0.4 to 0.5
3	Lupus erythematosus with hemolytic anemia and circulating anticoagulant	+	0.4 to 0.5
4	Chronic myelocytic leukemia with N.H.T.R.	±	0.6 to 0.8
5	Normal subject immunized to leukocytes	−	0.6 to 0.8
6	Chronic neutropenia	±	0.4 to 0.5
7	Acute myelogenous leukemia with N.H.T.R.	+	0.4 to 0.5
8	Leukemia, unclassified	+	0.4 to 0.5
9	Lupus erythematosus? with N.H.T.R.	−	0.6 to 0.8
10	Diagnosis? with N.H.T.R.	−	0.6 to 0.8
11	Diagnosis? with N.H.T.R.	−	0.6 to 0.8
12	Pancytopenia with N.H.T.R.	−	0.6 to 0.8
13	Chronic myelocytic leukemia	−	0.6 to 0.8
14	Chronic myeloid metaplasia	±	0.4 to 0.5
15	Primary hypersplenism with leukopenia	+	0.4 to 0.5
16	Monocytic leukemia	−	0.4 to 0.5
17	Granulocytopenia	4+	0.4 to 0.5
18	Aseptic meningitis and leukopenia	+	0.4 to 0.5
19	Acute leukemia with N.H.T.R.	+	0.4 to 0.5
20	N.H.T.R.	+	0.4 to 0.5

*N.H.T.R. = Nonhemolytic transfusion reaction.

RESULTS

The above materials and methods were applied to 2 major problems.

A. The Detection of Leukocyte Antibodies in Various Clinical States.—One-half milliliter of the leukocyte suspension from type O Rh-positive donors was incubated for 1 hour at 37° C. with an equal quantity of the test sera which was suspected of containing a leukocyte antibody. A portion of this cell suspension was used for slide agglutination tests. The remainder was placed in 10 ml. of Michaelis Universal buffer, pH 7.4, and the electrophoretic mobility of a number of single leukocytes was determined. In order to eliminate bias prior to performance of the leukoagglutinin and electrophoretic testing the samples of the suspect pathologic and normal sera were randomized and coded

by another individual so that the worker who actually performed these techniques had no prior knowledge of the contents of the tubes under study. Furthermore, the positive sera were retested at least twice against several other normal control sera at weekly intervals and once again several months later. The results of tests on 30 different normal sera and 20 suspected pathologic sera are shown in Table I. As can be seen, 13 out of 20 suspect sera produced leukocyte slowing. An equal number gave equivocal or definitely positive agglutinin reactions. However, in each group there was a positive result which could not be correlated with a similar result by the other method. The results obtained for the leukocyte electrophoresis method with both the normal control sera and suspected pathologic sera were consistent and reproducible. The same range of results was found with repetition of the tests on several occasions.

TABLE II. RESULTS OF TESTING SUSPECTED PATHOLOGIC SERA AND CONTROL SERA ON 2 OCCASIONS BY THE ELECTROPHORETIC TECHNIQUE

DATE	SUSPENDING MEDIA	ELECTROPHORETIC VELOCITY (μ/SEC./VOLT/CM.)
April 2, 1959	Saline	0.70
	Control serum	0.70
	Test serum (Patient No. 7)	0.50
April 6, 1959	Saline	0.70
	Control serum	0.70
	Test serum (Patient No. 7)	0.50

TABLE III. RESULTS OF TESTING SUSPECTED PATHOLOGIC SERA AND CONTROL SERA ON 3 SEPARATE OCCASIONS BY THE ELECTROPHORETIC TECHNIQUE

DATE	SUSPENDING MEDIA	ELECTROPHORETIC VELOCITY (μ/SEC./VOLT/CM.)
Aug. 28, 1959	Saline	0.72
	Serum (Patient No. 17)	0.43
Oct. 2, 1959	Saline	0.72
	Control serum	0.68
	Test serum (Patient No. 17)	0.50
Nov. 3, 1959	Control serum	0.70
	Test serum (Patient No. 17)	0.43

Tables II and III represent results obtained on 2 different sera by the electrophoretic technique on repeated testing.

A study of serial dilutions of several of the antisera showed electrophoretic slowing in the higher dilutions. However, the change in the end point was abrupt rather than gradual. This was distinctly different from that observed in the leukoagglutinin technique. This discrepancy in end point caused difficulty in correlation of these two methods.

B.—The second problem concerned the effect of sera containing antierythrocyte antibodies upon the leukocytes of donors of various blood groups.

One-half milliliter of fresh autologous and inactivated serum from a type O Rh-positive donor containing an anti-A saline titer of 1:4,096 and immune titer of 1:20,480 was incubated for 1 hour with equal volumes of leukocyte suspensions from individuals of A and O red cell blood groups. Slide agglutination tests were performed and the electrophoretic velocities of the serum-treated leukocytes were determined. The results are shown in Table IV. No

detectable agglutination of the leukocytes was evident either grossly or microscopically. A slowing of the electrophoretic velocity of the leukocytes was not produced by the serum on either set of cells.

Similar studies were performed with the use of high titer anti-B serum, leukocytes from individuals of B and O red cell groups, high titer anti-Rh (D) serum, and leukocytes from persons of O Rh-positive and O Rh-negative (D) erythrocyte blood groups. Agglutination was not discernible in any of these combinations, nor was a retardation of the electrophoretic mobility present (Tables V and VI).

TABLE IV. ELECTROPHORETIC MOBILITY OF LEUKOCYTES FROM GROUPS OF TYPE A AND O DONORS FOLLOWING INCUBATION WITH HYPERIMMUNE ANTI-A SERUM

	ANTISERUM PLUS LEUKOCYTES (A DONOR)		ANTISERUM PLUS LEUKOCYTES (O DONOR)	
CELL SUSPENSION MEDIA	MEAN (μ/SEC./VOLT/CM.)	STANDARD DEVIATION	MEAN (μ/SEC./VOLT/CM.)	STANDARD DEVIATION
Fresh autologous serum	0.76	0.052	0.63	0.052
56° C. inactivated serum	0.72	0.045	0.61	0.042
Saline	0.75	0.055	0.61	0.042

TABLE V. ELECTROPHORETIC MOBILITY OF LEUKOCYTES FROM GROUPS OF TYPE B AND O DONORS FOLLOWING INCUBATION WITH HYPERIMMUNE ANTI-B SERUM

	ANTISERUM PLUS LEUKOCYTES (B DONOR)		ANTISERUM PLUS LEUKOCYTES (O DONOR)	
CELL SUSPENSION MEDIA	MEAN (μ/SEC./VOLT/CM.)	STANDARD DEVIATION	MEAN (μ/SEC./VOLT/CM.)	STANDARD DEVIATION
Fresh autologous serum	0.65	0.55	0.73	0.052
56° C. inactivated serum	0.65	0.055	0.76	0.052
Saline	0.63	0.052	0.72	0.045

TABLE VI. ELECTROPHORETIC MOBILITY OF LEUKOCYTES FROM D-POSITIVE AND D-NEGATIVE DONORS FOLLOWING INCUBATION AT 37° C. WITH HIGH TITER ANTI-D SERUM

	SERUM PLUS O-POSITIVE CELLS		SERUM PLUS O-NEGATIVE CELLS	
CELL SUSPENSION MEDIA	MEAN (μ/SEC./VOLT/CM.)	STANDARD DEVIATION	MEAN (μ/SEC./VOLT/CM.)	STANDARD DEVIATION
Fresh autologous serum	0.7	0.0	0.61	0.042
56° C. inactivated serum	0.7	0.0	0.63	0.052
Saline	0.65	0.257	0.67	0.05

DISCUSSION

The electrophoretic method has recently been shown to be able to detect experimentally produced leukocyte antibody[1] by the observation of slowing of the electrophoretic velocity of leukocyte treated with such sera. The first phase of the present study concerned an evaluation of this method in the detection of leukocyte antibody present in clinical states. Out of a total of 20 sera from

patients suspected of having leukocyte antibody, 13 showed positive results with this technique. These results correlated well with those obtained by the agglutinin method. The advantages of the electrophoretic method which were discussed in a previous paper[1] include its specificity and the precise results obtained. The knowledge acquired in clinical problems with this method would tend to support our satisfaction with this mode of detection of leukocyte antibody. As donor cells from another individual were used as the test cells in our study, no comment can be made as to whether the leukocyte antibodies were also autoimmune in nature.

Confusion exists in reference to cross-incompatibility between immune anti-A, anti-B, anti-Rh (D) sera and leukocytes from donors of specific A, B, O, and Rh configurations. Frequent but inconstant leukocyte agglutinations in both compatible and incompatible combinations have been previously reported. Moeschlin and Schmid[2] examined 380 cross-matches performed with the serum and leukocytes of 30 patients. An agglutination of the leukocytes occurred in a third of the cases in which agglutination of the erythrocytes was demonstrated on the basis of the blood groups. Goudsmit and Van Loghem[3] tested the sera of 100 healthy donors against the leukocytes from type O individuals and found weakly positive agglutinations in 10 instances. On the other hand, Dausset and associates[4] matched sera of 300 normal donors with ABO and Rh compatible leukocyte suspensions and failed to note any agglutination. Butler[5] had difficulty in differentiating specific and nonspecific clumping in his agglutination studies. He found many mixed cell clumps and attributed some agglutination reactions to erythrophagocytic activity rather than to true leukocyte agglutinations. He concluded that there is no relationship between leukocyte agglutinins and ABO blood groups. A similar conclusion is suggested by the work of Bakemeier and Swisher.[6] Confronted with this divergence of reports, the clarification of the ABO problem utilizing the electrophoretic method was attempted. It would appear from our observations that no relation exists between the leukocytes of specific A, B, O, and Rh donors and their corresponding antisera, tending to rule out the possibility that these antigens are present on the white cell surface. The importance of this observation is more than academic. It also permits the use of leukocytes from donors of any blood group as test cells in this technique.

SUMMARY

The electrophoretic method for the detection of leukocyte antibody was applied to several clinical problems. It was found that antibody could be detected by this technique in 13 out of 20 of the sera of patients suspected of having this abnormality. This correlated well with the leukoagglutinin technique. Investigation of the leukocytes of individuals with specific A, B, O, and Rh configurations exposed to the sera containing various erythrocyte antibody revealed no change in their surface charge.

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STUDIES OF A WALDENSTRÖM-TYPE MACROGLOBULIN WITH RHEUMATOID FACTOR PROPERTIES

JULIUS KRITZMAN, M.D., HENRY G. KUNKEL, M.D., JOHN MCCARTHY, AND ROBERT C. MELLORS, M.D.
BOSTON, MASS., AND NEW YORK, N. Y.

A macroglobulin obtained from the blood of a patient with typical Waldenström's macroglobulinemia has been shown to have serologic properties similar to those of rheumatoid factor. In other respects (behavior in the ultracentrifuge, reaction with rabbit antimacroglobulin serum, cryoprecipitation, electrophoretic mobility, and carbohydrate content) it was a typical macroglobulin. An important difference between it and rheumatoid factor is its pH optimum of about 6.0; the optimum for rheumatoid factor serologic activity is about 8.6. This difference in pH optimum affects both the precipitin reaction with aggregated gamma globulin and the agglutination of various test particles. The macroglobulin was demonstrated in the cytoplasm of spleen and lymph node cells by the use of fluorescent-aggregated gamma globulin. Extracts of splenic tissue (obtained at autopsy) had the same serologic activity as the serum factor. The morphology of the cells of origin of this protein is described in detail.

The patient from whom this protein was obtained had no joint symptoms at any time despite the tremendous amount of protein with rheumatoid factor–like activity. The possibility is raised that all Waldenström macroglobulins may have biologic activity related to the antibody produced by the cell from which they were derived.

THE possibility that myeloma proteins and Waldenström macroglobulins might possess biologic activities has long been considered. Since these proteins are closely related to normal constituents of the gamma globulin fraction which contain antibodies, they might well also represent antibodies.[1, 2] The concept has been proposed recently that the cells producing these proteins are somatic mutations of individual antibody-producing cells[3-5] that might retain some of the specificity in their protein products of the original antibodies. Current evidence strongly suggests that the rheumatoid factors represent a group of closely related antibodies with physical, chemical, and antigenic properties which closely resemble known antibodies of the 19S class.[6, 7] It is of special interest, therefore, to have encountered a typical case of Waldenström's macroglobulinemia in which the pathologic protein demonstrated many properties which were similar though not identical to typical rheumatoid factors.

From the Arthur G. Rotch Laboratory of the Boston Dispensary, and the Department of Medicine, Tufts University School of Medicine, Boston, Mass.; The Rockefeller Institute, and The Hospital for Special Surgery, New York, N. Y.

Supported in part by a research grant from the National Institute of Arthritis and Metabolic Disease, National Institutes of Health, Bethesda, Md.

Received for publication July 15, 1960.

CASE REPORT

A 59-year-old bus maintenance worker* (N.E.C.H. No. 126-093) was admitted to The New England Center Hospital on April 4, 1959, for evaluation of progressive easy fatigability of one year's duration.

Fatigue had been his only symptom, but after admission to the hospital he suddenly experienced shaking chills, fever, left anterior chest pain with nonproductive cough, and dyspnea. He had been treated at home with intramuscular penicillin and other antibiotics, and, though the fever subsided promptly, he continued to complain of cough, chest pain, dyspnea, extreme weakness, easy fatigability, and drenching sweats in the afternoon and night. Because of these persistent symptoms, he was hospitalized elsewhere. A chest x-ray showed a density at the left lung base, interpreted as unresolved pneumonia. Antibiotic therapy was continued and bronchoscopy was performed. One month before admission to New England Center Hospital, he was discharged with symptoms unchanged, and an additional symptom of persistent marked hoarseness following negative bronchoscopy. He denied joint symptoms but stated that for about the past 6 months he had had frequent mild epistaxes. His past history revealed that at age 29 he had had syphilis, which was treated for one year with intravenous and intramuscular medication. Blood and spinal fluid tests for syphilis remained negative after treatment. His average daily alcohol intake had been 3 quarts of beer and occasional whiskey. He had smoked one package of cigarettes daily for 40 years.

Physical examination revealed a well-developed, well-nourished, but chronically ill–appearing white man. The vital signs were normal. Examination of the optic fundi revealed marked distention of the retinal veins, and there were numerous hemorrhages and exudates bilaterally. There was crusted blood in both nares but no hemorrhagic lesions were noted in the oral mucous membranes. The heart was not enlarged and there were no significant murmurs present. There was a moderate increase in the anteroposterior diameter of the chest, the chest was hyperresonant to percussion, and there were expiratory wheezes scattered throughout both lung fields. There was dullness to percussion at the left lung base posteriorly, and the breath sounds were decreased in this area. Abdominal examination revealed that the liver and spleen were not only enlarged downward, but were quite bulky and protuberant. The liver edge was felt approximately 4 cm. below the right costal margin, and the spleen was palpable 8 cm. below the left costal margin. There was no lymphadenopathy. At time of admission no petechiae or ecchymoses were noted on the skin. A shower of petechiae appeared on both legs during the patient's stay in the hospital and disappeared within 48 hours.

The volume of packed red cells was 32 per cent. The hemoglobin was 10.7 Gm. per cent. The white blood count was 6,800 per cubic millimeter. The differential count was as follows: polymorphonuclear leukocytes, 25 per cent, lymphocytes, 73 per cent, monocytes, 2 per cent, plasma cells, 1 per cent. Ninety per cent of the lymphocytes were atypical in that many of them had irregular borders and an unusually large amount of cytoplasm. Some resembled plasma cells. There was marked rouleaux formation by the red cells, but no morphologic red cell abnormalities were noted. Bone marrow examination revealed normocellular marrow with moderate infiltration with "lymphocytoid" cells, such as were seen in the peripheral blood. These cellular changes will be described in greater detail. The Westergren sedimentation rate was 139 mm. per hour. The blood Hinton test was negative. The total serum protein level was 7.4 Gm. per cent, with 2.1 Gm. per cent albumin and 5.3 Gm. per cent globulin. A BSP test showed 25 per cent retention in one hour. Several urinalyses showed a faint trace of proteinuria, but repeated tests for Bence Jones protein were negative. The one stage prothrombin time was 36 per cent, and became 48 per cent after 3 days of parenteral vitamin K therapy. The Lee-White clotting time in glass was 17 minutes. Bleeding time determinations, platelet counts, and tourniquet tests were normal. A direct Coombs test was negative. The indirect Coombs test on one occasion was positive against fresh donor cells, but was negative against donor cells that were over 2 hours old.

*We are grateful to Dr. Justin L. Richman for having referred this case to one of us (J. K.).

The patient's blood type was A positive. Chest x-ray showed bilateral bullous emphysema and blunting of the left costophrenic angle which was consistent with thickened pleura caused by previous pneumonitis. A flat film of the abdomen confirmed the hepatosplenomegaly; upper gastrointestinal series was negative for esophageal varices or other defects. Skull x-ray and x-ray survey of the long bones were negative for bone defects or lesions.

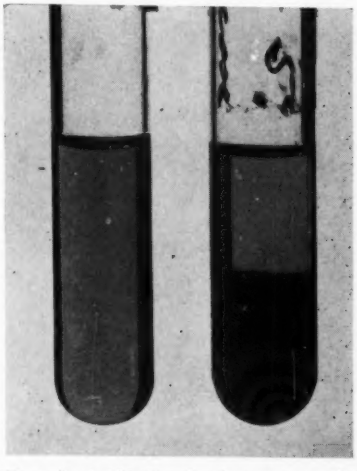


Fig. 1.—Serum of patient at 4° and at 37° C. A viscous cryoglobulin is apparent on the right in the 4° C. specimen.

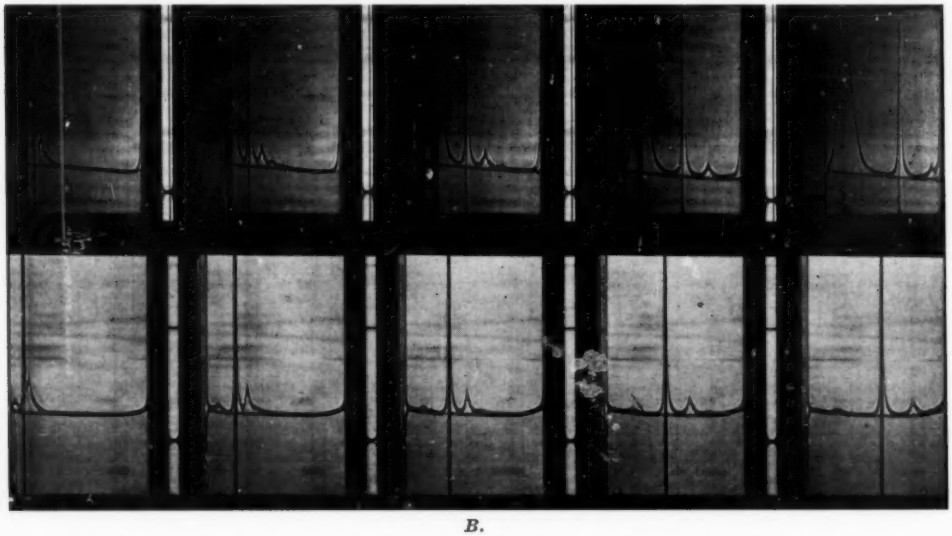
Paper electrophoresis of the patient's serum showed a very striking elevation of the gamma globulin fraction (Fig. 3). Periodic acid–Schiff stain showed this to be rich in carbohydrate. The homogeneous nature of the gamma globulin increase suggested multiple myeloma, but in view of the absence of bone symptoms or bone lesions and in view of the "lymphocytoid" appearance of the cells in the marrow and peripheral blood, Waldenström's macroglobulinemia seemed a more likely diagnosis. The analytical ultracentrifugation (to be described) confirmed the suspected presence in the patient's serum of large amounts of 18.5S material. Treatment was started with chlorambucil, 6 mg. daily, and the patient was transfused with 2 units of whole blood. After 10 days of treatment there was a definite decrease in the hemorrhages and exudates in the eye grounds. The profuse sweating of which the patient complained also decreased. In 2 weeks the eye grounds were clear and remained so throughout the course of his illness. He was able to return to work for the first time in 5 months. Chlorambucil therapy was stopped after one month, when the white count had fallen to 4,500. Two weeks after chlorambucil therapy was stopped it was 9,200, and the differential percentages were then: polymorphonuclear leukocytes, 82, lymphocytes, 16, monocytes, 2. The volume of packed red cells was 37 per cent. Approximately 6 weeks after therapy had been discontinued, the patient again complained of drenching sweats, easy fatigue, weakness, and some nasal bleeding. Chlorambucil was started again at a daily dose of 4 mg., but after 10 days he felt no better, and his white count had fallen to 3,500. Dexamethasone, 0.75 mg. 3 times daily, was started, and there was rapid symptomatic improvement. The improvement was maintained on an average dose of 0.375 mg. dexamethasone twice a day until approximately Nov. 15, 1959. At this time he noted the return of fatigue, anorexia, sweats, and hoarseness. The hematocrit had gradually fallen to 33 per cent, but there had been no change in either the white blood or differential count. Because these symptoms failed to respond to an increase in dexamethasone dosage, he was readmitted to The New England Center Hospital on Dec. 17, 1959.

Physical examination showed no change other than one fresh hemorrhage in the left fundus. Shortly after admission the subject developed pain in the region of the maxillary sinuses, an intermittent purulent nasal discharge, and daily rectal temperature elevations to as high as 103° F.

Culture of the nasal discharge showed a predominance of pneumococci sensitive to penicillin. Treatment with local measures for improvement of drainage, and intramuscular administration of 500,000 units penicillin every 6 hours, caused a marked decrease in nasal symptoms but did not affect the daily fever. Blood cultures were negative. One sputum culture showed *Staphylococcus aureus* resistant to penicillin, but chest x-rays showed no evidence of pneumonitis, and there was no change in physical findings in the chest. It was thought that the fever might be due to the underlying disease, and treatment with chlorambucil, 6 mg. daily, was again started. On the tenth hospital day the temperature suddenly rose to 105° F., the blood pressure fell to 70/40, and pulse rate rose to 140 per minute. He became rapidly moribund despite oxygen and maintenance on intravenous levarterenol and corticosteroids. On the assumption that he probably had an overwhelming infection with *Staphylococcus aureus,* he was treated with large doses of intramuscular chloramphenicol and intravenous erythromycin.

During the final 24 hours dullness to percussion over the left lower half of the chest was noted. He died on Dec. 28, 1959. The principal findings during autopsy were the presence of a sterile pneumonic consolidation of the left lower lobe, marked hepatosplenomegaly, and mesenteric lymphadenopathy. A portion of the spleen and some of the lymph nodes were frozen for special study.*

A.



B.

Fig. 2.—*A*, Ultracentrifuge patterns of the whole serum. *B*, Isolated macroglobulin fraction. Centrifugation from left to right. Frames taken at 8, 12, 24, and 32 minutes.

SPECIAL STUDIES

1. Physical Properties of Serum and Isolated Fractions.—Fig. 1 shows the distinct cryoglobulin that was present in the serum. This readily became miscible when the temperature of the serum was raised to 37° C. The cryoglobulin was not a true precipitate but a viscous opalescent material which settled to the bottom of the tube. Removal of this material showed considerable enrichment of macroglobulin, although there still was contamination by other serum proteins to approximately 50 per cent. The viscous material was difficult to wash, and further purification was carried out by zone electrophoresis. Fig. 2 shows the ultracentrifuge pattern of the whole serum and of the isolated macroglobulin fraction. Only a small amount of slowly sedimenting material remained. These patterns demonstrate the principal 18.5S component as well as small amounts of 28S and 36S components. These studies were performed in a Spinco analytic ultracentrifuge at 20° C. at 52,640 r.p.m.

*We are grateful to Dr. Stavros E. Meimaridis of the Department of Pathology, The New England Center Hospital, for these tissues.

Electrophoretic analysis on filter paper and in a starch supporting medium demonstrated that the macroglobulin was a gamma globulin migrating at pH 8.6 in barbital buffer only slightly faster than the normal gamma globulin peak. Fig. 3 shows the paper electrophoresis pattern.† Carbohydrate staining[8] indicated a high carbohydrate content similar to that of other macroglobulins, but quantitative assays were not carried out.

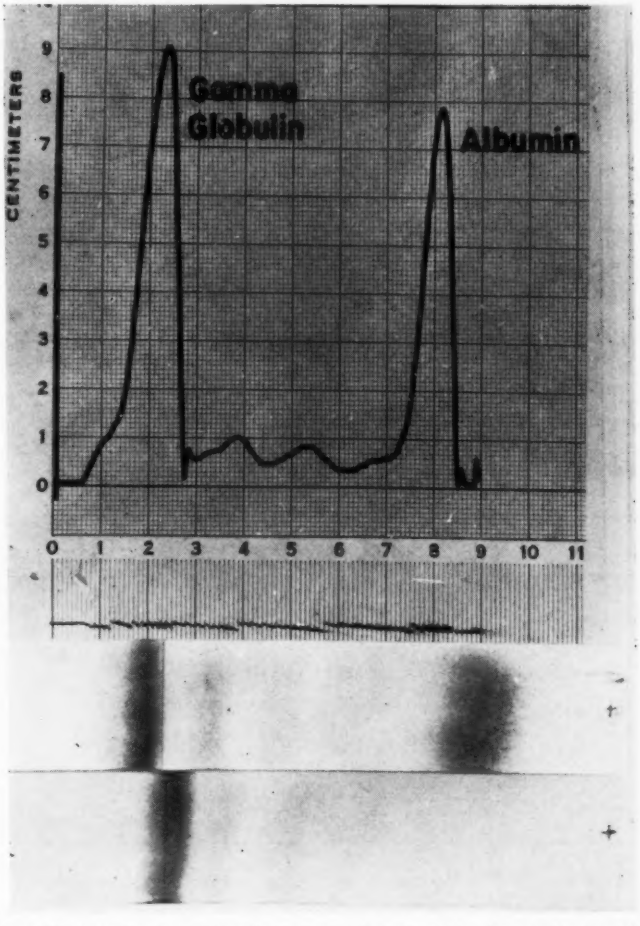


Fig. 3.—Densitometer pattern of patient's serum. Lower strip demonstrates the strongly positive P.A.S. reaction of the gamma globulin fraction.

2. Precipitin Reaction With Aggregated Gamma Globulin.—The precipitin studies were performed by standard techniques.[9] When the reaction was carried out in buffer, the precipitates were washed in the same buffer. The most striking finding with this serum was its strong precipitin reactivity with aggregated gamma globulin. No other serum examined in these laboratories showed activity at such high dilutions. A $1/500$ dilution of serum still gave a distinct immediate precipitate in capillary tubes. This activity was proved to be associated with the pathologic macroglobulin in a variety of ways. Density gradient ultracentrifugation[2] showed all activity in the bottom macroglobulin fractions. The highly purified preparation shown in Fig. 2 had the same activity per milligram of macroglobulin as did the serum. After addition of aggregated gamma globulin to the purified preparation, 95 per cent of the 18.5S peak and all of the visible 28S peak was removed in the precipitate.

†The densitometry was performed by Eli Dubinsky of the Chemistry Laboratory of The New England Center Hospital.

This experiment showed that all of the pathologic macroglobulin possessed the property of reacting with aggregated gamma globulin. Finally, analysis of the precipitate formed on the addition of the aggregates showed the presence of the 18.5S component as well as the 26S material (Fig. 4). For this study the precipitate was dissolved in glycine-HCl buffer (pH 3.4) prior to centrifugation. This pattern differed from those obtained with rheumatoid serum in the very small amount of 7S material present.

Fig. 4.—Ultracentrifuge patterns of the dissolved specific precipitate between soluble aggregates of gamma globulin and patient's serum. The first two frames show the heterogeneous aggregate. Frames taken at 6, 8, 12, and 16 minutes.

TABLE I. EFFECT OF PH ON TITER OF AGGLUTINATION OF CHROMIC CHLORIDE–TREATED CELLS BY PATIENT'S SERUM AND RHEUMATOID ARTHRITIS SERA

BUFFER USED FOR SERIAL DILUTION	PATIENT'S SERUM	RHEUMATOID ARTHRITIS SERA	
		NO. 1	NO. 2
Phosphate pH 5.2	1 : 32,768	1 : 16	1 : 1,280
Phosphate pH 5.7	1 : 32,768	1 : 4	1 : 1,280
Phosphate pH 6.8	1 : 32,768	1 : 4	1 : 2,560
Phosphate pH 7.4	1 : 2,048	1 : 16	1 : 5,120
Phosphate pH 8.0	1 : 128	1 : 64	1 : 20,480
Veronal pH 8.6	1 : 16	1 : 32	1 : 1,280

Fig. 5 illustrates a precipitin curve with the purified preparation of macroglobulin. This material behaved like other macroglobulins in being readily dissociable with sulfhydril compounds. This was observed in the ultracentrifuge with formation of monomeric units of approximately 7S sedimentation constant. With this dissociation all precipitating activity disappeared, as is shown in Fig. 5. This was absent either after dialysis against iodoacetamide or in the presence of excess SH reagent.

In the course of these studies it was noted that the precipitin reaction was very sensitive to pH changes. No reaction was obtained in barbital or glycine HCl buffer at pH 8.6. Fig. 6 illustrates precipitin curves at 3 different pH levels and shows the marked reduction of precipitate at higher pH. Crude, heated gamma globulin was used in these experiments rather than the purified aggregates previously employed. The pH effect is also illustrated in Table I, where one of the serologic tests is utilized at various pH levels and constant ionic strength. The point of maximum titer for the two rheumatoid arthritis sera was at pH 8.0, a point where this patient's serum showed very low reactivity.

3. Pattern of Reactivity in Different Serologic Tests for Rheumatoid Factor.—Table II summarizes the results of a large number of experiments utilizing this serum and isolated

fractions in the various serologic tests. The most striking positive test obtained was in the chromic chloride–treated red cell system. This test has usually correlated well with the latex fixation reaction.[10] This test was carried out as previously described using human group O red blood cells coated with 1 per cent gamma globulin. Control elements for the slide test included washed, uncoated cells and cells treated with 1 per cent bovine albumin solutions and various concentrations of chromic chloride in isotonic saline. This serum had the highest titer of any serum encountered. The ordinary latex fixation test by the original method of

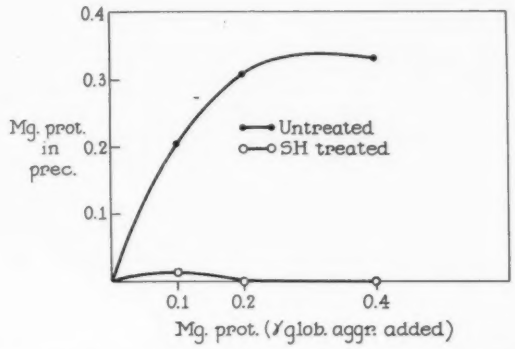


Fig. 5.—Precipitin curve of soluble aggregate with patient's serum before and after treatment with mercaptoethanol.

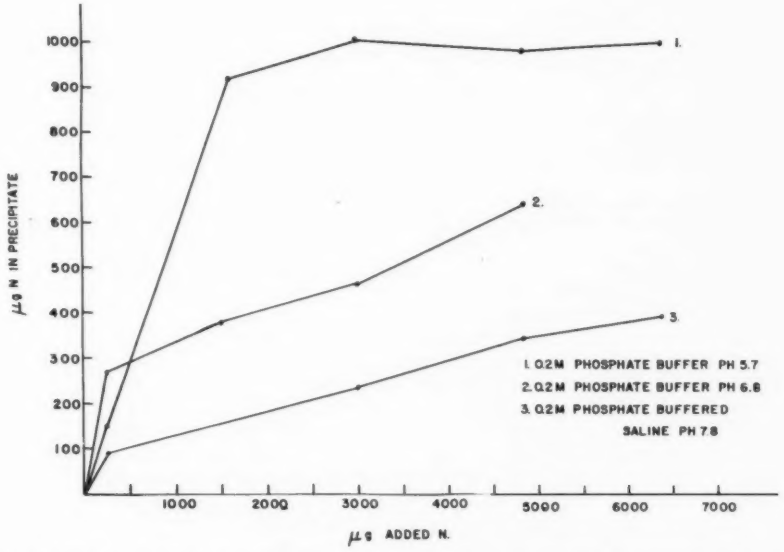


Fig. 6.—Precipitin curves of patient's serum and crude aggregated gamma globulin showing pH effect.

Singer and Plotz[11] was completely negative. However, in view of the pH dependence of this protein in regard to its reactivity with aggregated gamma globulin, the usual buffer used to dilute the serum would preclude a positive reaction. Attempts were therefore made to carry out the latex test in saline. However, when this was done, considerable spontaneous agglutination occurred, even in absence of the serum. This agglutination by the added gamma globulin alone was not inhibited by the patient's serum, although strongly inhibited by normal serum.

When latex particles were coated with purified aggregates of gamma globulin and then washed, preparations were obtained in some instances which did not agglutinate in saline alone or in normal serum. The serum of this patient, as well as the isolated macroglobulin, agglutinated these latex particles strongly. Rheumatoid arthritis sera which gave positive ordinary latex fixation tests also gave positive reactions with the aggregate-coated particles.

TABLE II. AGGLUTINATION TESTS WITH PATIENT'S SERUM

TEST	RESULTS
1. Chromic chloride–treated red cell coated with gamma globulin	Positive to $1:2.5 \times 10^6$
2. Latex fixation test with whole serum*	Negative
3. Latex fixation test with latex coated with aggregated gamma globulin	Positive to 1:10, 240
4. Latex inhibition test	Positive
5. Test vs. Rh-positive cells coated with anti-Rh sera	Positive to 1:20, 480
6. Sensitized sheep cell test	Negative
7. Tanned cell test (coated with gamma globulin)*	Negative

*Usual buffers were employed.

TABLE III. DIFFERENTIAL COUNT OF NUCLEATED CELLS IN PATIENT'S BONE MARROW*

CELL TYPE	PATIENT'S MARROW	NORMAL MARROW (WINTROBE)[23]
Myeloblasts	0.8%	0.3 to 5.0
Promyelocytes	0.6%	1.0 to 8.0
Myelocytes	12.2%	5 to 25
Metamyelocytes	9.2%	13 to 32
Polymorphonuclear leukocytes	3.4%	7 to 30
Basophils	0	0 to 0.7
Lymphocytes	37.6	3 to 17.0
"Atypical" lymphocytes	14.6	0
Plasma cells	2.0	0 to 2.0
Monocytes	0	0.5 to 5.0
Megakaryocytes	1.0	0.03 to 3.0
Pronormoblasts	0.6%	1.0 to 8.0
Normoblasts	18.0	7.0 to 32

*Five hundred cells were counted. Counts were repeated twice several days apart and agreed very well.

An inhibition reaction utilizing a positive rheumatoid arthritis serum showed a lack of inhibition by the patient's serum similar to that of rheumatoid arthritis sera.

The sensitized sheep cell test[12] and the tanned cells coated with gamma globulin showed negative reactions. The latter was to be expected in view of the high pH of the buffer used to dilute the serum. This test was not studied with other buffers or saline.

Reaction With Red Cells Coated With Incomplete Anti-Rh Antibodies.—This test, originally described by Waller and Vaughan[14] and Grubb and Laurell,[15] was studied in some detail in regard to the reaction with this serum as compared with that of rheumatoid arthritis sera. It has recently been demonstrated[16] that different Rh antibodies on Rh-positive red cells give very different patterns of reactivity with different rheumatoid arthritis sera. Rh coat Murphy,* which has been found to react with all sera giving positive latex fixation tests, also reacted with the serum of this patient at high dilutions. Rh coat Ripley† also gave a positive reaction at similar dilutions. A few other Rh coats also gave positive reactions, but the majority, which were highly reactive with certain rheumatoid arthritis sera, were entirely negative with this serum.

4. Studies of the Cells Producing the Macroglobulins.—The bone marrow and even the peripheral blood of this patient showed the presence of large numbers of unusual cells that

*Knickerbocker Blood Bank.
†Furnished by Dr. Marion Waller.

were difficult to classify into either the lymphocyte or plasma cell series. Some were fairly typical lymphocytes in that they ranged from approximately 9 to 14 μ in diameter (by comparison with neighboring red cells), and had round nuclei with densely clumped chromatin and scanty rims of deep blue cytoplasm. In Table III these are referred to simply as lymphocytes. In addition, however, there were other cells which in general were 1½ to 2 times the size of the lymphocytes, had eccentric nuclei which had densely clumped chromatin separated by clear areas, and had large amounts of cytoplasm. The cytoplasm of many of these cells appeared vacuolated. In some there was a definite perinuclear clear zone, so that there was a marked resemblance to plasma cells. Because of their resemblance to cells seen

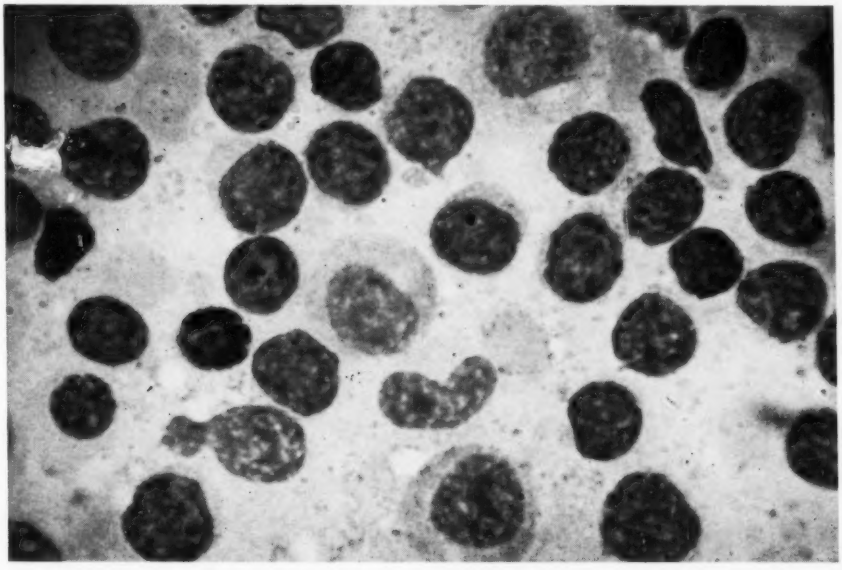


Fig. 7.—Bone marrow smear showing infiltration by lymphocytes having typical scanty rims of cytoplasm and "atypical lymphocytes" with eccentric nuclei, large amounts of cytoplasm, and occasional vacuoles.

in infectious mononucleosis, we have called them atypical lymphocytes (Table III). The majority of the lymphocytes seen in the patient's peripheral blood before treatment was started were of the atypical type. After treatment with chlorambucil was started the majority of the lymphocytes were small, typical cells. Despite this cellular change, samples of serum removed for study throughout the illness showed no change in the content or behavior of the protein. Fig. 7 shows both types of cells in the bone marrow. Fig. 8 shows two atypical lymphocytes in the peripheral blood.

Frozen sections of lymph node and spleen were treated with fluorescent aggregated human gamma globulin by techniques described previously.[17] After washing, brilliant fluorescence of the cells described above was observed. Fig. 9 illustrates the cytoplasmic fluorescence from one section of lymph node. No nuclear fluorescence was observed. This fluorescence was blocked by pretreatment with anti-19S gamma globulin antiserum. The aggregated gamma globulin did not cause fluorescence of control sections. The fluorescence observed with the tissues of this patient exceeded that noted previously in cases of rheumatoid arthritis.[17]

5. Spleen and Lymph Node Studies.—Portions of the patient's spleen and some of the mesenteric lymph nodes were frozen immediately after autopsy. Aliquots of spleen, weighing about 100 Gm., were extracted by the method of Abrams, Cohen, and Meyer.[18] The frozen tissue was thawed, minced with a Stadie blade, and washed repeatedly until the washings

gave a negative test for protein and had no agglutinating activity against gamma globulin–coated cells. The washed tissue was then homogenized in cold, buffered 0.07 M KCl, and the homogenate was centrifuged at 14,000 r.p.m., 4° C. for one hour. This caused separation into 3 layers: a clear, supernatant layer, a jelly-like adherent layer, and a layer of nuclei and cellular debris. Each layer was dialyzed for 48 hours against 25 per cent dextran in 0.8 per cent saline. The resulting solutions were tested against cells coated with gamma globulin. The clear supernatant fraction gave an agglutination titer of 1:32 and the second layer a titer of 1:512. There was insufficient fluid in the cell layer for testing.

6. Antigenic Properties of the Macroglobulin.—Three rabbits were injected with the highly purified macroglobulin preparation illustrated in Fig. 2, *B*. Two responded with high titer antibodies after a booster injection. Quantitative precipitin curves with these antisera indicated a strong reaction with the macroglobulin obtained from this patient. Two heterologous macroglobulins obtained from patients with Waldenström's macroglobulinemia reacted strongly with this antiserum, as did preparations of normal 19S gamma globulin and rheumatoid factor. Much weaker reactions were obtained with ordinary 7S gamma globulin per milligram of Ag added.

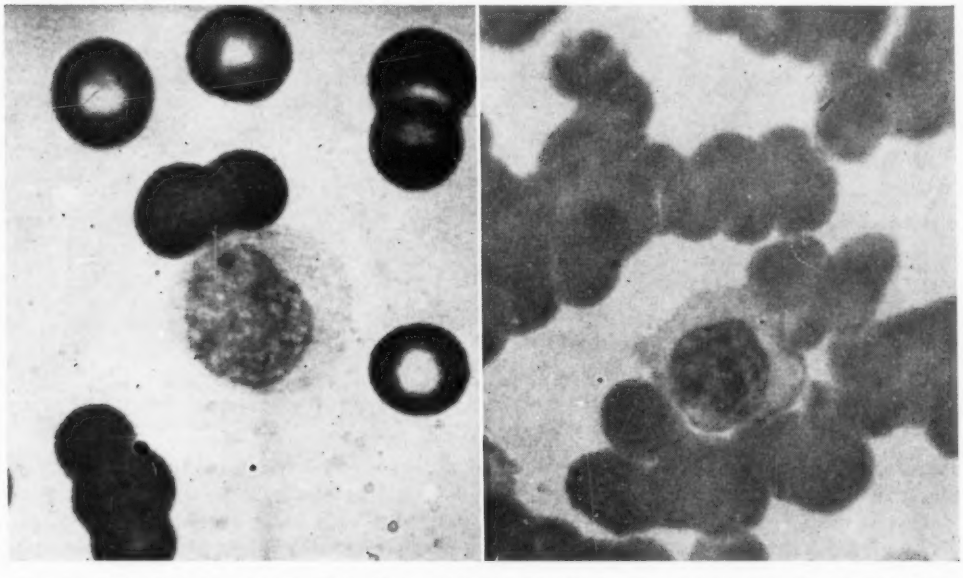


Fig. 8.—"Atypical lymphocytes" in the peripheral blood showing large amount of cytoplasm with some vacuoles.

DISCUSSION

A number of different rheumatoid factors have been isolated by ion exchange chromatography.[19, 20] However, it seems unlikely that a type identical with the protein described above has been encountered. Certainly, no similar type has been observed in the experience of the authors. The major difference in the behavior of this protein from that of other rheumatoid factors was its unusual pH optimum for biologic activity. This resulted in negative results with the usual latex fixation test because of the use of alkaline buffers. Other tests for rheumatoid factor activity employing saline or phosphate buffers at lower pH gave highly positive results. The precipitin reaction with soluble

aggregates of gamma globulin was strikingly positive. This finding suggests that by testing other sera containing acquired macroglobulins over a wide pH range additional examples of antibody-like protein–protein interactions may be discovered.

In chemical and physical properties this macroglobulin was very similar to others isolated from patients with Waldenström's macroglobulinemia. In the ultracentrifuge, in addition to the main 19S, the usual accompanying 28S and 34S components were clearly visible. These macroglobulins were readily dissociable with sulfhydril compounds with complete loss of biologic activity. Antigenically, the macroglobulin components were similar to those in other cases of Waldenström's macroglobulinemia. The only essential difference was in the specific biologic activity, which was very similar to rheumatoid factors and which was not possessed by a wide variety of other Waldenström-type macroglobulins that were tested.

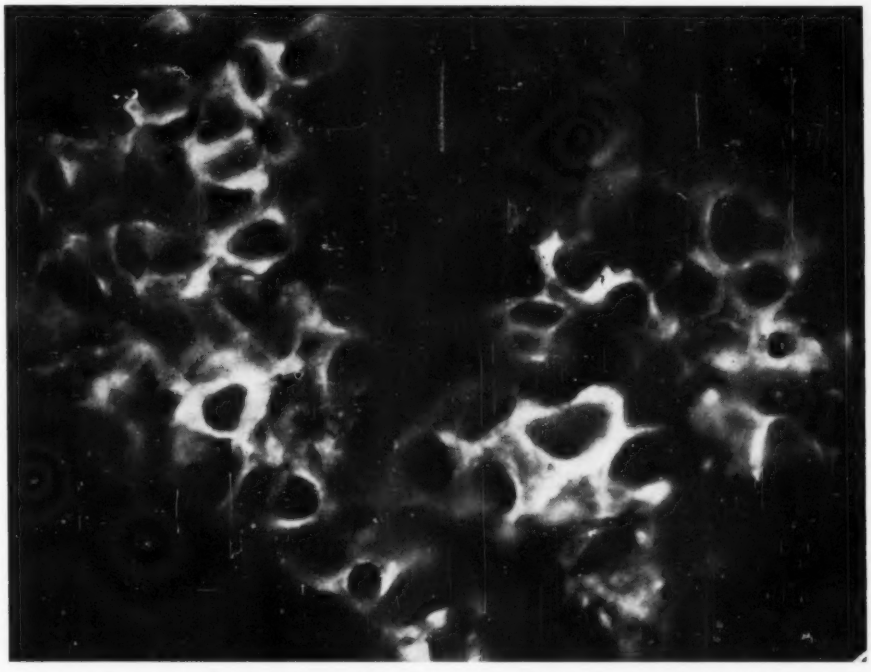


Fig. 9—Fluorescence in the cytoplasm of lymph node cells after treatment of the section with fluorescent aggregated gamma globulin. (×1,000.)

The abnormal cells that were observed in bone marrow, lymph node, spleen, and peripheral blood were demonstrated by the fluorescent labeling technique to be the source of the pathologic macroglobulin. The agglutinating activity of the spleen extracts confirmed this. Many of these cells had scanty cytoplasm and resembled the "lymphocytoid cells" classically associated with macroglobulinemia. Others showed considerably more cytoplasm and appeared to be of the plasma cell series. Both types showed fluorescence of the cytoplasm. No nuclear fluorescence of the type recently reported[21] was observed.

The absence of arthritis is not surprising but emphasizes the unlikelihood of the pathogenic significance of rheumatoid factors. Many reports of rheumatoid factors in conditions without arthritis have recently appeared.[22] As in this case, these frequently do not show positive sheep cell agglutination. The case demonstrates that tremendous amounts of this type of factor may circulate in the body without producing joint lesions or symptoms.

The rheumatoid factor activity of this abnormal protein, produced in tremendous amounts by this patient with Waldenström's macroglobulinemia, represents another biologic activity for these proteins. Cold agglutinin[2] and complement fixation[3] activities have been described previously for certain of the pathologic proteins of this disease. It seems possible that they all may possess activities similar to known antibodies. However, in most instances, the right antigen has not been found for testing. The question is certainly raised again as to whether the neoplastic proliferation in this disease arises through somatic mutation of single antibody producing cells and results in cells still capable of producing protein containing some of the specificity of the original antibody.

SUMMARY

A case of typical Waldenström's macroglobulinemia is described in which the pathologic macroglobulin showed biologic activity similar to rheumatoid factors. The protein showed physical, chemical, and antigenic properties very similar to those of the macroglobulins seen in other cases of Waldenström's activity. The resemblance to rheumatoid factors was striking in certain of the serologic tests. Difference in pH optima was observed which caused negative tests where alkaline buffers were employed. The fluorescent labeling technique demonstrated the site of origin of the pathologic protein.

Addendum.—Recently, 2 additional Waldenström macroglobulins have been encountered[24] where the pathologic protein possessed rheumatoid factor–like properties.

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OBSERVATIONS ON NORETHANDROLONE-INDUCED ABNORMALITIES IN PLASMA DECAY OF SULFOBROMOPHTHALEIN AND INDOCYANINE GREEN

CARROLL M. LEEVY, M.D., GILBERT R. CHERRICK, M.D.,* AND CHARLES S. DAVIDSON, M.D.
JERSEY CITY, N. J., AND BOSTON, MASS.

The mechanism of norethandrolone-induced sulfobromophthalein retention has been investigated by study of selected aspects of the metabolism of this dye in fourteen healthy subjects and three patients with fatty liver. Abnormal sulfobromophthalein retention provided a more sensitive index to the subcellular lesion induced by norethandrolone than indocyanine green, serum transaminase, alkaline phosphatase, serum bilirubin, cephalin cholesterol flocculation, or hippuric acid synthesis.

Plasma sulfobromophthalein decay studies after administration of 100 to 150 mg. of norethandrolone daily for 7 to 12 days demonstrated a decrease in initial dye removal from an average of 14.2 to 10.3 per cent per minute, and an increase in 45 minute retention from an average of 2.0 to 13.8 per cent in healthy subjects. In patients with fatty liver, although norethandrolone administration was accompanied by rapid restoration of normal histology on light microscopy, the initial plasma decay of sulfobromophthalein was reduced 47 per cent, and 45 minute retention increased fourfold.

Functional or morphologic changes, besides a cholangiolar lesion, appear to contribute to sulfobromophthalein abnormalities after norethandrolone. This drug had no demonstrable effect on extrahepatic removal or amino acid conjugation of this dye.

ABNORMAL sulfobromophthalein (BSP) retention has been regularly noted after administration of norethandrolone.†[1, 2] This BSP abnormality could be due to an excretory defect secondary to morphologic or metabolic changes which interfere with uptake, conjugation, or biliary excretion of the dye. Atrophy of the microvilli of bile canaliculi after norethandrolone administration has been observed by electron microscopy,[3] although light microscopy and biochemical liver function tests other than BSP excretion may remain unchanged. The present study was undertaken to investigate further the mechanism of BSP

From the Thorndike Memorial Laboratory and the Second and Fourth (Harvard) Medical Services, Boston City Hospital; the Department of Medicine, Harvard Medical School, Boston, Mass.; and the Division of Hepatic Metabolism and Nutrition, Department of Medicine, Jersey City Medical Center, and Seton Hall College of Medicine, Jersey City, N. J.

Supported in part by the United States Army Medical Research and Development Command, Department of the Army, under Contract No. DA-49-193-MD-2013, and in part by grants from the Nutrition Foundation, Inc., New York; Merck & Co., Inc., Rahway, N. J., and the National Heart Institute, Public Health Service Grant H-4530(RI).

Received for publication Aug. 25, 1960.

*Public Health Service Research Fellow of the National Heart Institute. Present address: Nemazee Hospital, Shiraz Medical Center, Shiraz, Iran.

†Norethandrolone (Nilevar) supplied by G. D. Searle & Co., Chicago, Ill.

retention after norethandrolone. Effects of this drug on BSP metabolism, including its extrahepatic removal, excretion as a conjugate in bile, and plasma decay patterns before and after treatment were studied. Comparison was made of the influence of norethandrolone on removal kinetics of BSP and indocyanine green (ICG).

MATERIALS AND METHODS

Fourteen patients without clinical or laboratory evidence of liver disease and three with severely fatty liver were studied. Each of the subjects was given 100 to 150 mg. of norethandrolone daily for 7 to 12 days and several aspects of BSP metabolism studied before and after receipt of the drug. The drug was given orally in divided doses to thirteen subjects and intramuscularly to one subject, E. E., who had previously had a partial gastrectomy. A series of liver function tests including serum bilirubin,[4] serum alkaline phosphatase,[5] serum glutamic oxalacetic transaminase,[6] serum albumin and globulin,[7] cephalin flocculation,[8] and thymol turbidity[9] was obtained before and after norethandrolone. Serial percutaneous liver biopsies were performed with the Vim-Silverman needle in the patients with fatty liver and in eight of the normal subjects.

TABLE I. CHROMATOGRAPHY OF BILE FOR BSP FOLLOWING ITS INJECTION BEFORE AND AT THE COMPLETION OF NORETHANDROLONE ADMINISTRATION

PATIENT NO.	SULFOBROMOPHTHALEIN	BEFORE NORETHANDROLONE		AFTER NORETHANDROLONE	
		MG./100 ML.	PER CENT	MG./100 ML.	PER CENT
1	Free	0.524	41.5	0.335	36.7
	Conjugated	0.740	58.5	0.578	63.3
	Total amount	1.264	100.0	0.913	100.0
3	Free	0.317	46.2	0.277	41.4
	Conjugated	0.370	53.8	0.392	58.6
	Total amount	0.687	100.0	0.669	100.0

TABLE II. HIPPURIC ACID SYNTHESIS FOLLOWING ADMINISTRATION OF 1.77 GM. SODIUM BENZOATE I.V. BEFORE AND AFTER NORETHANDROLONE

PATIENT NO.	BEFORE NORETHANDROLONE		AFTER NORETHANDROLONE	
	HOUR	GM.	HOUR	GM.
1	1	0.75	1	0.80
	2 }	0.15	2	
	3 }		3	0.32
2	1	1.4	1	1.1
	2 }	0.15	2	
	3 }		3	0.25
3	1	0.99	1	0.83
	2 }	0.18	2	
	3 }		3	0.29
4	1	0.92	1	1.11
	2 }	0.21	2	
	3 }		3	0.31

The effects of norethandrolone on the skeletal muscle uptake of BSP were studied in three normal subjects by evaluation of left femoral arteriovenous dye differences during a constant intravenous infusion of 3.0 mg. of the dye per square meter of body surface area per minute before and after receipt of the drug. Quantitation of renal excretion of BSP was obtained in four normal subjects before and after the drug by collection of all urine excreted for 2 hours after receipt of a single intravenous injection of 5 mg. of the dye per kilogram of body weight.

TABLE III. COMPARISON OF LIVER FUNCTION TESTS AND INDOCYANINE GREEN BEFORE AND AFTER

PATIENT NO.	DOSAGE OF NORETHANDROLONE AND PERIOD OF ADMINISTRATION		SERUM BILIRUBIN (MG. PER CENT)	ALKALINE PHOSPHATASE (BESSEY-LOWRY UNITS)	SERUM GLUTAMIC OXALACETIC TRANSAMINASE UNITS	THYMOL TURBIDITY (MACLAGAN UNITS)	CEPHALIN FLOCCULATION
1	100 mg. daily for 9 days	Before	0.3	0.7	16	2.0	2+
		After	0.4	1.0	30	0.8	2+
2	120 mg. daily for 8 days	Before	0.6	1.0	36	0.5	1+
		After	0.4	1.5	24	0.8	2+
3	150 mg. (I.M.) daily for 9 days	Before	0.5	2.5	18	2.2	2+
		After	0.3	1.6	6	2.0	2+
4	120 mg. daily for 12 days	Before	0.4	1.2	12	1.2	1+
		After	0.5	1.6	10	1.4	2+
5	120 mg. daily for 7 days	Before	0.6	1.5	12	2.9	1+
		After	0.7	1.6	6	2.1	1+
6	100 mg. daily for 9 days	Before	0.6	1.8	20	2.8	2+
		After	0.4	1.2	10	2.6	2+
7	120 mg. daily for 7 days	Before	0.6	1.3	10	2.0	2+
		After	0.8	1.6	14	2.2	2+

Mean ± Standard deviation.— Before
After

Bile was obtained for chromatography by duodenal intubation before and after administration of norethandrolone to two normal subjects. Ascending chromatography of the bile which contained BSP was carried out in propyl alcohol : water : acetic acid (10 : 5 : 1 volume per volume).[10] The BSP was eluted from filter paper into 1 normal sodium chloride and was read at 580 mμ on the Beckman DU spectrophotometer. Hippuric acid synthesis was studied in four normal subjects before and after administration of the drug. In this procedure 1.7 Gm. of sodium benzoate was given intravenously and urine was collected through a 3 hour period.[11] Hippuric acid was extracted from the urine with ether in a continuous extraction apparatus. The residue obtained by distilling off the ether was treated with bromine and sodium hypobromite to destroy traces of urea. Hippuric acid nitrogen was then determined in the residue by the micro-Kjeldahl procedure.[12]

Plasma decay of BSP was studied in eight normal subjects and the three patients with chronic alcoholism and hepatic steatosis before and after administration of norethandrolone. Five milligrams per kilogram of body weight of BSP was given as a single rapid intravenous injection. Venous samples were taken at 3 and 5 minutes from the time of dye injection and at 5 minute intervals thereafter for 1 hour. Decay patterns were also studied after administration of 2 mg. BSP per kilogram of body weight to the patients with fatty liver. Plasma removal rates of indocyanine green (ICG) using 0.5, 1.0, or 2.0 mg. of this dye per kilogram of body weight were investigated in the normal subjects.[13] Serum BSP concentration was determined by alkalinization and reading in an Evelyn colorimeter at 580 mμ. Readings of plasma ICG concentration were made in a Beckman DU spectrophotometer at 815 mμ.

RESULTS

No untoward symptoms or clinical evidences of jaundice, hair changes or other androgenic effects, hepatomegaly, or fluid accumulation were noted during administration of norethandrolone. Body weight increased an average of 2 pounds during receipt of the drug without an appreciable change in food intake. A comparison of liver function tests and histology by light microscopy in normal subjects before and after norethandrolone showed no significant change.

AND PLASMA REMOVAL OF SULFOBROMOPHTHALEIN
NORETHANDROLONE IN 7 NORMAL SUBJECTS

SULFOBROMOPHTHALEIN (5.0 MG. PER KILOGRAM BODY WEIGHT)			INDOCYANINE GREEN (0.5 TO 2.0 MG. PER KILOGRAM BODY WEIGHT)			
INITIAL RATE OF DECAY (PER CENT PER MINUTE)	HALF-LIFE (MINUTES)	PER CENT RETAINED AFTER 45 MINUTES	DOSAGE	INITIAL RATE OF DECAY (PER CENT PER MINUTE)	HALF-LIFE (MINUTES)	PER CENT RETAINED AFTER 20 MINUTES
16.9	3.8	1.3	1.0	22.0	3.0	2.0
11.1	6.0	18.0	1.0	23.6	3.0	1.2
16.0	4.0	1.3	0.5	20.8	3.0	3.4
5.3	9.0	10.0	1.0	19.6	5.0	3.8
14.4	4.4	0.4-	2.0	18.7	2.5	4.3
11.2	11.0	11.6	2.0	18.0	2.5	2.1
12.7	5.1	1.2	1.0	20.1	3.1	2.2
10.5	6.5	17.0	1.0	17.1	4.0	4.3
15.0	3.5	3.4	0.5	19.5	3.5	5.0
11.0	10.5	9.1	0.5	19.6	3.0	3.3
14.0	4.0	4.4	0.5	15.6	4.2	4.5
11.1	5.0	14.5	0.5	18.8	2.0	2.0
13.1	5.0	2.8	0.5	15.4	3.5	4.9
9.2	8.0	16.6	0.5	19.5	2.5	4.0
14.5 (±1.5)	4.3 (±0.2)	2.1 (±1.4)		18.8 (±2.8)	3.2 (±0.5)	3.7 (±1.2)
9.9 (±2.1)	9.0 (±2.5)	13.8 (±3.6)		19.4 (±2.0)	3.1 (±1.0)	2.9 (±1.2)
$P < .05$	$P < .05$	$P < .05$				

Norethandrolone did not influence extrahepatic uptake or renal excretion of BSP. There was an average initial skeletal muscle uptake of BSP by the left lower extremity of .001 ± .0005 mg. per minute both before and after this drug was given. An average of 2 ± 0.5 per cent of administered dye appeared in the urine at both times. Extraction ratio and estimated hepatic blood flow showed no change with norethandrolone.

Chromatography of bile into which BSP had been excreted showed similar pre- and postnorethandrolone patterns (Table I). The relative amount of dye excreted as a conjugate was slightly increased after receipt of the drug. Hippuric acid synthesis was not altered, two of the subjects exhibited a slight increase and two a decrease in the amount of hippuric acid recovered (Table II).

The plasma decay curves of BSP and ICG in normal subjects before norethandrolone was given could be divided into two parts. Initially, decay was exponential, the falling concentration being a straight line when plotted on semilogarithmic paper. The average rate of decay during the exponential segment was 14.2 (S.D. 2.0) per cent per minute for BSP as compared with 18.8 (S.D. 2.8) per cent per minute for ICG (Table III). Following this, deceleration of decay occurred with both dyes so that after 45 minutes 2.0 per cent (S.D. 1.4) of the initial concentration of BSP and at 20 minutes 3.7 per cent (S.D. 1.2) of the initial concentration of ICG were present. After administration of norethandrolone the initial rate of decay of BSP decreased from 14.2 per cent (S.D. 2.0) per minute to 10.3 per cent (S.D. 2.6) per minute, but the ICG curves showed no significant alteration. The half-life of BSP increased from 4.3 (S.D. : .2) to 9.0 minutes (S.D. 2.5), while that of ICG remained the same. The mean postnorethandrolone BSP level 45 minutes after injection was 13.8 per cent (S.D. 3.6) of the initial concentration, whereas, after drug therapy,

TABLE IV. EFFECT OF 120 MG. OF NORETHANDROLONE DAILY FOR 10 DAYS ON HISTOLOGY AND SULFOBROMOPHTHALEIN RETENTION IN FATTY LIVER OF THE ALCOHOLIC

PATIENT NO.	HISTOLOGY		SERUM BILIRUBIN (MG. PER CENT)	ALKALINE PHOSPHATASE (BODANSKY UNITS)	CEPHALIN FLOCCULATION	SERUM GLUTAMIC OXALACETIC TRANSAMINASE UNITS	SERUM GLUTAMIC PYRUVIC TRANSAMINASE UNITS	SULFOBROMOPHTHALEIN	
								INITIAL RATE OF DECAY (PER CENT PER MINUTE)	PER CENT RETAINED AFTER 45 MINUTES
12	Severe fatty liver	Before	0.1	4.7	Neg.	58	21	6.8	8.0
	Normal liver	After	0.0	5.0	Neg.	46	58	4.8	31.0
13	Severe fatty liver	Before	0.1	6.3	Neg.	28	50	7.5	6.5
	Normal liver	After	0.1	4.8	Neg.	14	58	5.6	26
14	Severe fatty liver	Before	0.1	2.6	Neg.	28	21	11.9	2
	Normal liver	After	0.1	3.6	Neg.	29	36	3.4	25
							Average.—		
							Before	8.7	5.5
							After	4.6	27.3

the level of the ICG 20 minutes after injection was 2.9 per cent (S.D. 1.2) of the initial concentration. Mean values did not differ when the amount of ICG was raised to 1 or 2 mg. per kilogram of body weight (Table III).

Prior to norethandrolone administration the plasma decay of BSP in the patients with alcoholism and fatty liver (by liver biopsy) showed a decrease in initial removal rate with normal or slightly increased 45 minute dye concentrations. Following the period of norethandrolone administration liver biopsy in each instance was interpreted as normal by light microscopy (Fig. 1).

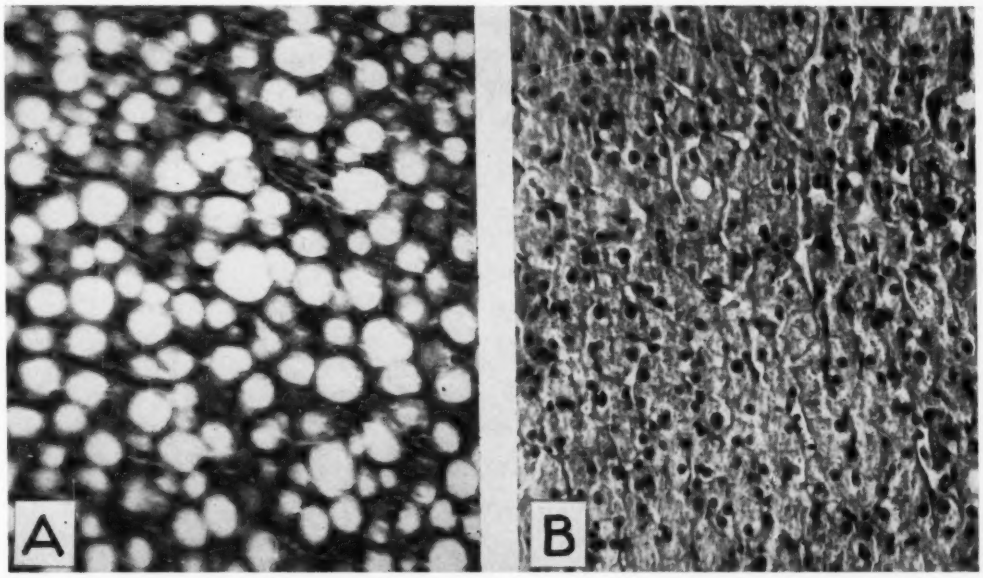

Fig. 1.—*A*, Severe fatty liver, Patient No. 14. (×80; reduced ⅕.) *B*, Liver, now normal, after 10 days of general diet and norethandrolone. (×80; reduced ⅕.)

After norethandrolone administration plasma decay of BSP was markedly abnormal in each instance (Table IV), with an average reduction in the initial removal rate from 8.7 to 4.6 per cent per minute (47 per cent decrease) and an average increase in retention after 45 minutes from 5.5 to 27.3 per cent per minute (396 per cent increase). Abnormal plasma decay of BSP was observed in both the 2 mg. and 5 mg. dosage of the dye (Fig. 2). BSP plasma decay repeated 10 days after discontinuation of norethandrolone was normal.

DISCUSSION

A major problem with BSP testing has been abnormal dye retention in the absence of clinical, histologic, and other biochemical evidence of liver disease. This has been attributed to subclinical liver disease, which implies that subcellular morphologic changes might be responsible.[14] Identification of a lesion by electron microscopy in patients with norethandrolone-induced BSP retention and normal histology by light microscopy confirms the concept in this instance.[3] The demonstration that continued administration of this drug in high dosage

ultimately leads to intrahepatic cholestasis accompanied by an increase in serum bilirubin, transaminase, and alkaline phosphatase, indicates the potential progressive nature of the lesion.[15]

From our studies it is evident that neither interference with renal excretion or a skeletal muscle uptake, nor an abnormality of amino acid conjugation is responsible for the observed BSP alterations. The major change in plasma decay of BSP following receipt of norethandrolone occurs in the second or decelerated portion of the curve, theoretically reflecting alteration of biliary excretion of the dye.[16] In normal subjects and in patients with fatty liver, norethandrolone also reduced the initial disappearance rate of BSP. This suggests the presence of a functional or morphologic lesion which interferes with transport of BSP from sinusoid to parenchymal cell. Estimations of hepatic

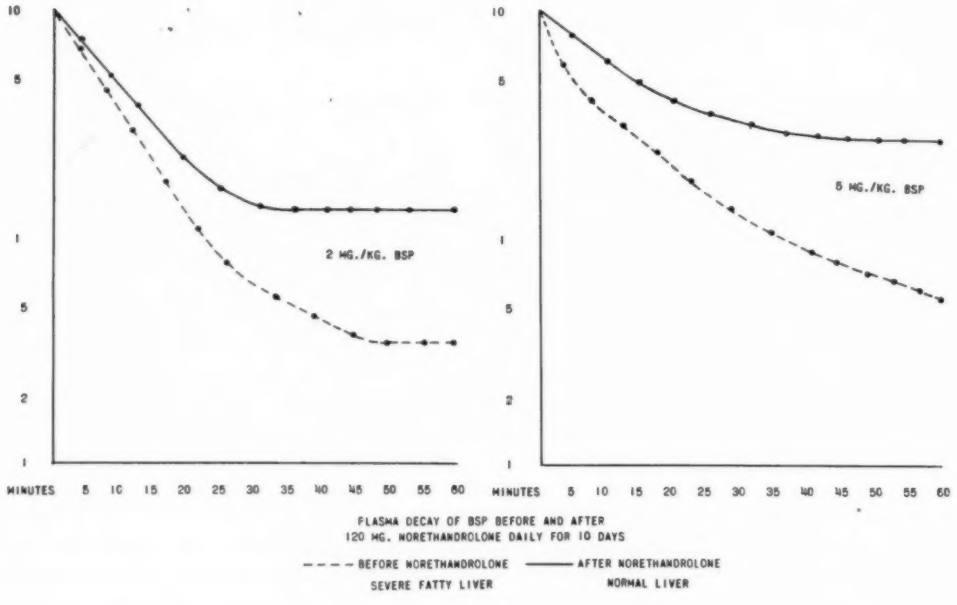

Fig. 2.

storage of BSP and its transport from hepatic parenchymal storage into bile[17] after norethandrolone show a depression of both values and do not contribute additional information on the mechanism of BSP retention. In our experience effect of norethandrolone on the hepatic extraction ratio of BSP is correlated with the degree of dye retention induced by this drug. Mild degrees of BSP retention are not associated with alteration of the extraction ratio; a significant decrease occurs with retention of more than 25 per cent of the dye.[18]

Abnormal plasma decay of BSP following receipt of norethandrolone was not accompanied by a similar change in plasma decay of ICG. This is not a dosage phenomenon, as simultaneous administration of 2 mg. of both dyes per kilogram of body weight produces BSP retention without affecting ICG removal. ICG appears to provide a more specific index to hepatic excretory function than

BSP since it has negligible extrahepatic removal and is not excreted as an amino acid conjugate.[10, 13] Failure of norethandrolone to influence ICG excretion suggests that either ICG is a less sensitive indicator of canalicular disease or that a canalicular lesion alone is not responsible for the BSP retention. Against the first alternative is the fact that patients with biliary obstruction may show a lessened removal of ICG; while plasma decay of ICG is more rapid than that of BSP in patients with mild parenchymal cell dysfunction.[13]

The simultaneous improvement in histology by light microscopy and decrease of hepatic removal capacity of BSP which follows receipt of norethandrolone is unique. Discovery of a pharmacologic agent with similar anabolic potency which does not produce structural or functional hepatic lesions is desirable. An evaluation of plasma decay of BSP as an index to hepatotoxicity provides a yardstick for clinical screening of such compounds.

SUMMARY AND CONCLUSIONS

1. Investigation of the mechanism of norethandrolone-induced sulfobromophthalein (BSP) retention indicates this drug has no effect on extrahepatic removal or amino acid conjugation of this dye. In dosage of from 100 to 150 mg. daily for from 7 to 12 days, the drug caused a significant decrease in initial plasma decay of BSP, attributed primarily to alteration in hepatic uptake of the dye, and an increase in 45 minute retention of BSP, reflecting the presence of an excretory defect. It had no effect on plasma removal of indocyanine green (ICG).

2. BSP provides a more sensitive index than ICG, serum transaminase, alkaline phosphatase, or serum bilirubin to the canalicular lesion and excretory defect produced by norethandrolone. Functional or morphologic lesions besides those previously described appear to contribute to the BSP abnormalities which followed norethandrolone administration.

3. Treatment of patients with alcoholism and a severely fatty liver (by biopsy) with norethandrolone and a general hospital diet was associated with rapid restoration of normal histology on light microscopy, although initial plasma decay of BSP was reduced 47 per cent, and 45 minute retention increased fourfold. BSP abnormality in these patients was the only reflection of the subcellular lesion produced by this drug.

Grateful acknowledgment is made of the advice and help of Dr. Rudi Schmid and Dr. Maceo M. Howard, and the technical aid provided by Lois Betten, Lydia Hammaker, and Jean Muenzen.

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CHROMIC PHOSPHATE DISAPPEARANCE IN CIRRHOSIS OF THE LIVER AND CONGESTIVE HEART FAILURE

CHEVES McC. SMYTHE, M.D.*
CHARLESTON, S. C.

Disappearance rates of colloidal chromic phosphate in patients with cirrhosis of the liver, congestive heart failure, and miscellaneous diseases of the hepatobiliary system have been compared with those in normal human beings. When these disappearance constants are used for calculation of hepatic blood flow, rates of flow approximating those obtained by other methods are found in normal individuals. There was no change of disappearance rates in the presence of severe hepatocellular disease. Other variables must be involved in determination of colloid disappearance rates which might invalidate their use for study of hepatic perfusion in clinical hepatocellular disease.

NOT the least of the reasons behind the increased knowledge of circulatory dynamics is the advance in techniques which allows study of perfusion rates of a single organ or vascular bed by indirect methods which disturb the subject being studied relatively little. The technique of following the removal rates of various radioactive colloids introduced by Dobson and Jones[1] in 1951 as a method of measuring blood flow through the liver has the enormous advantage of requiring much less manipulation of the patient than the bromsulphalein (BSP) removal method of Bradley.[2]

Dobson and Jones[1] have presented the theoretical discussion of the assumptions by which hepatic blood flow is estimated from colloidal disappearance rates and data which indicate that radioactively labeled colloidal chromic phosphate is a suitable colloid.[3] In addition to a large amount of experimental work done in animals, Dobson has published results in a group of normal young men studied by this method. This paper presents the results obtained in patients with various hepatobiliary disturbances.

Calculation of Hepatic Blood Flow.—Division of the natural logarithm of 2 by the half time ($T\frac{1}{2}$) gives one a constant k, expressing the disappearance slope $k = \frac{0.693}{T\frac{1}{2}}$ or fraction of the blood volume cleared per minute. This constant k is then multiplied by the blood volume (BV) to determine blood flow (BF); thus BF = kBV. The half time is read from a semilogarithmic plot of the experimentally determined disappearance slope.[1]

From the Department of Medicine and the Medical College Hospital, Medical College of South Carolina, Charleston, S. C.

Supported by Research Grant No. AH-985, United States Public Health Service.
Received for publication Sept. 6, 1960.

*John and Mary R. Markle Scholar in Medicine.

TABLE I. CHROMIC PHOSPHATE DISAPPEARANCE IN CONVALESCENT HOSPITAL PATIENTS

CONTROL PATIENT NO.	AGE	SEX	BODY WEIGHT (KILOGRAMS)	CORRECTED HEMATOCRIT (%)	BLOOD VOLUME (ML.)	T½ (MINUTES)	k	HEPATIC BLOOD FLOW (L./MINUTE)	DIAGNOSIS
1	32	M				1.4	0.49		Peptic ulcer
2	32	M	58.5	35.1	5,940	2.2	0.32	1.9	Convalescent pneumonia
3	56	M	88.4	36.4	5,090	2.5	0.28	1.4	Parkinsonism
4	62	M	71.2	46.6	4,850	2.0	0.34	1.7	Postoperative hernia
5	37	M	64.8	35.1	4,810	1.6	0.43	2.1	Convalescent pneumonia
6	46	M	78.9	43.9	6,350	2.0	0.34	2.2	Gastritis
7	72	M		28.3	3,320	1.1	0.63	2.1	Osteoarthritis
8	21	M	79.4	39.2	5,740	1.8	0.39	2.2	Erythema multiforme
9	31	M	69.8	31.5	4,890	1.9	0.37	1.8	Convalescent subarachnoid hemorrhage
10	62	M	79.8	31.1	4,370	1.7	0.41	1.8	Peptic ulcer
11	28	F	66.2	35.1	3,230	1.1	0.63	2.0	Rheumatic fever
12	37	F	83.4	35.1	3,270	1.5	0.46	1.5	Stasis ulcer of leg
13	56	F		39.6	3,940	1.4	0.49	2.0	Mild hypertension
					Mean	1.7	0.43	1.9	
					S. D. ±	0.41	0.11	0.2	

METHODS

The patients were fasting. An inlying Cournand needle was placed in a brachial vein or artery and a sample of blood was drawn for background count. Approximately 15 μc of P^{32} as colloidal chromic phosphate and 2.5 mg. of T-1824 in 10 ml. of saline were injected intravenously in the opposite arm.* After 2 minutes, blood samples were obtained every minute for 5 minutes, and then every 2 minutes for 10 more minutes. Two milliliter aliquots of whole blood were pipetted into standard paper planchets and counted with a Geiger-Müller tube. Values were plotted on semilogarithmic paper and the best slope fitted visually. Half time was taken from this slope. Characteristically, the last 3 or 4 points on the curve do not fit the initial slope. No attempt to modify the initial slope by treatment of these points was made, since they are thought to characterize the behavior of a smaller colloid not selectively removed by the liver (Fig. 1). T-1824 concentrations were measured in a Coleman Junior spectrophotometer, and the plasma volume calculated from the dilution of a single sample taken at 11 minutes after the injection of the dye. Hematocrits (Wintrobe) were corrected for trapped plasma and venous/total body ratio by multiplication of observed values by 0.9. No effort was made to calculate splanchnic volume.

TABLE II. DISAPPEARANCE CONSTANTS WITH VARIOUS RADIOACTIVE COLLOIDS IN NORMAL MAN

AUTHOR	NUMBER OF CASES (N)	$T\frac{1}{2}$ (MINUTE)	k	ESTIMATED HEPATIC BLOOD FLOW (L./ MINUTES)	COLLOID
Dobson and associates[3]	29	2.3	0.282	1.8	Chromic phosphate
Vetter and associates[4]	25	2.6	0.262	1.3	Gold
Krook[5]	30	4.4	0.158	0.7	Gold
Riddell[6]	18	4.9	0.140	1.6	Gold
Fauvert[9]	22	4.2	0.166		Gold
Halpern[8]	5	3.1	0.226	1.1	Iodinated albumin
Smythe[11]	18	3.3	0.22	1.2	Iodinated albumin
Smythe	13	1.7	0.43	1.9	Chromic phosphate
Waldstein[7]				1.6	Mean of reported PSP removal data

RESULTS

Results are presented in tabular form. The means at the bottom of each table are not entirely valid since in the pathologic states there were varying degrees of disturbance of normal physiology. In the "diagnosis" column in each table, an effort is made to note these clinical variations. This note is based on the clinical status of the patient at the time of the determination as judged by history, physical examination, laboratory data, and hospital course.

The data from the 13 normal control subjects—hospitalized convalescent patients—are set out in Table I; Fig. 1 is an example of a normal disappearance curve. Mean disappearance time was 1.7 minutes ± S. D. 0.41 minute. The mean disappearance constant (k) calculated from this half time was 0.43 ± S. D. 0.11. Mean hepatic blood flow calculated from this constant was 1.9 L. per minute ± 0.2 L. per minute. For those patients with accurate weights this amounts to a mean of 0.025 L. per kilogram per minute. Mean blood volume for the 10 patients in whom it was calculated was 65.8 ml. per kilogram. There is considerable variation in the data. Half time varied from 1.1 to 2.5 minutes, k from

*The colloidal chromic phosphate was prepared in Dr. Dobson's laboratory and given to us through his great kindness.

TABLE III. CHROMIC PHOSPHATE DISAPPEARANCE IN CIRRHOSIS OF THE LIVER

PATIENT NO.	AGE	SEX	BODY WEIGHT (KILOGRAM)	CORRECTED HEMATOCRIT (%)	BLOOD VOLUME (ML.)	$T_{1/2}$ (MINUTES)	k	HEPATIC BLOOD FLOW (L./MINUTE)	DIAGNOSIS	CLINICAL CONDITION
1	48	F	42.6	34.2		1.6	0.43		Alcoholic cirrhosis	Hepatomegaly; ascites
2	53	M	60.8	39.6	6,360	1.4	0.49	3.2	Alcoholic cirrhosis	Hepatomegaly; portal hypertension; hepatoma
3	48	M	90.2	44.1	7,260	3.4	0.20	1.5	Alcoholic cirrhosis	Jaundice; ascites; hepatomegaly; portal hypertension
4	40	M	95.3	28.8	8,990	2.4	0.29	2.6	Alcoholic cirrhosis	Ascites; hepatomegaly
5	51	M	70.3	39.6	6,610	2.6	0.27	1.8	Alcoholic cirrhosis	Jaundice; hepatomegaly
6	43	F	47.6	29.2	4,210	1.8	0.39	1.6	Alcoholic cirrhosis	Hepatomegaly
7	52	F	50.8	24.8	4,610	2.0	0.35	1.6	Xanthomatosis Biliary cirrhosis	Jaundice; ascites; portal hypertension; hepatomegaly
8	61	M	46.2	24.7	5,080	1.2	0.58	2.9	Alcoholic cirrhosis	Ascites; hepatomegaly, portal hypertension
9	58	M	73.9	41.5	5,920	2.4	0.29	1.7	Alcoholic cirrhosis	Jaundice; ascites; hepatomegaly; hepatoma
10	54	F	55.4	36.0	4,550	3.0	0.23	1.1	Alcoholic cirrhosis	Jaundice; ascites; precoma
11	49	F	66.2	29.3	4,910	3.4	0.20	1.0	Postnecrotic cirrhosis	Ascites; portal hypertension
12	49	F	58.9	34.7	4,630	2.3	0.30	1.4	Postnecrotic cirrhosis	Ascites; portal hypertension; coma
13	30	F	63.5	35.5	5,420	2.7	0.26	1.4	Postnecrotic cirrhosis	Ascites; jaundice
					Mean	2.3	0.33	1.8		
					S. D. ±	0.68	0.11	0.7		

0.63 to 0.28, and the calculated hepatic blood flow from 1.4 to 2.2 L. per minute. No explanation for these variations is apparent, and they bear no overt relationship to the condition of the subjects.

The reported value for k is higher than that reported in other series (Table II). The steeper slopes indicate more rapid disappearance. Since incomplete extraction would decelerate rather than accelerate any disappearance slope, this cannot be a factor in the rapid disappearance rates. Failure to register counts occasioned by the method of counting is another possibility. Counting a beta emitter in a liquid medium results in the loss of counts. In 4 normal patients, counting rates from a dried film of 2 ml. of blood pipetted into a planchett

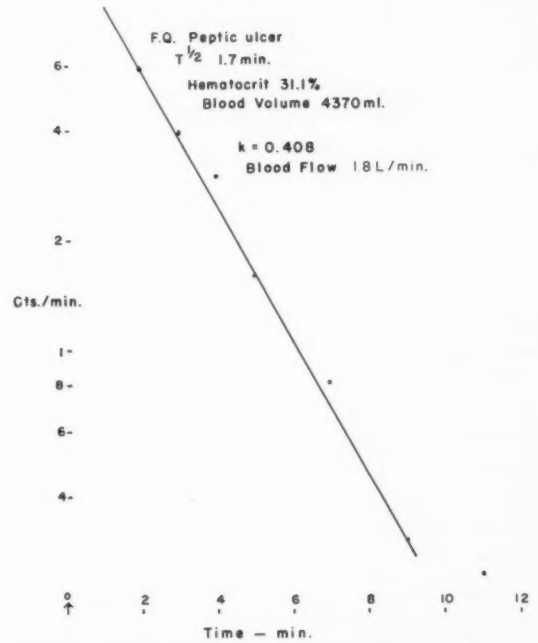

Fig. 1.—A normal disappearance curve in a man convalescent from a minor hemorrhage from a peptic ulcer. In this particular patient, half time, k, and blood flow are all very close to the means for the whole group of normal subjects.

were 43 per cent higher than those from identical samples counted in the liquid state. However, disappearance slopes did not vary significantly (mean, 0.1 minute) in these patients, as determined by dry and wet counting. The percentage of error when repeated counts were made on the same sample was ± 3 per cent.

Cirrhosis of the Liver (Table III).—The 14 observations (13 patients) recorded in the table are in patients with cirrhosis of a number of etiologies. Ten of the 13 had alcoholic liver disease, 2, postnecrotic cirrhosis, and 1, primary xanthomatous biliary cirrhosis. Mean half time was 2.3 minutes ± S. D. 0.68 minute. Fig. 2 is an example of slower disappearance curve. Mean disappearance constant was 0.33 ± S. D. 0.11, and hepatic blood flow was 1.8 L. per

minute ± S. D. 0.7. For 12 patients with satisfactory blood volume determination mean blood volume was 84.1 ml. per kilogram. Half time in the group of cases varied from 1.2 to 3.4 minutes. The patients with very severe disease, e. g., those in hepatic coma, did not necessarily have the slowest disapperance rates. The one patient tested twice had a faster chromic phosphate disappearance time on the morning of her death in hepatic coma than she did 2 months previously when she first was hospitalized with ascites (Fig. 2). Thus, a patient who has a sufficient loss of hepatocellular function to die in hepatic coma may have phagocytic cell function still intact. This emphasizes the concept of divergence of hepatocellular function and hepatic circulation so clearly expressed by Fauvert and associates.[10]

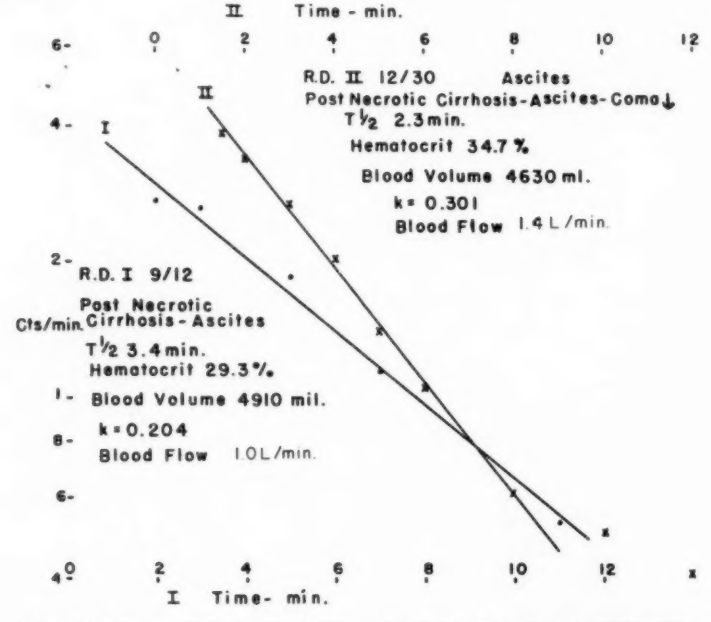

Fig. 2—The flatter disappearance slope illustrates slower disappearance rates in patients with cirrhosis. The steeper slope is taken from the same patient when she was in a terminal condition. This suggests that disappearance rates do not correlate well with other indices of hepatic function.

TABLE IV. DISAPPEARANCE CONSTANTS WITH VARIOUS RADIOACTIVE COLLOIDS IN CIRRHOTICS

AUTHOR	NUMBER OF CASES	T½ (MINUTES)	k	ESTIMATED HEPATIC BLOOD FLOW (L./MINUTE)	COLLOID
Vetter[4]	12	7.3	0.095	0.5	Gold
Krook[5]	33	7.5	0.092	0.5	Gold
Fauvert[9]	39	7.6	0.091		Gold
Halpern[8]	6	4.8	0.143	0.6	Iodinated albumin
Smythe[11]	9	4.6	0.170	1.0	Iodinated albumin
Smythe	13	2.3	0.33	1.9	Chromic phosphate
Bradley[12]	39			1.1	BSP removal

In Table IV previous experience of other authors with radiocolloids in patients with cirrhosis of the liver is summarized. The tabulated data suggest the possibility that the radiocolloid used seems to have more to do with the rate

of removal of the substance than anything else. All authors report lower blood flow and slower disappearance rates in the cirrhotic patients than in the normal individuals. There is no treatment of the data which attempts to interpret the disappearance rates as a phagocytic function which might be altered in liver disease rather than as a change dependent upon only decreased hepatic perfusion.

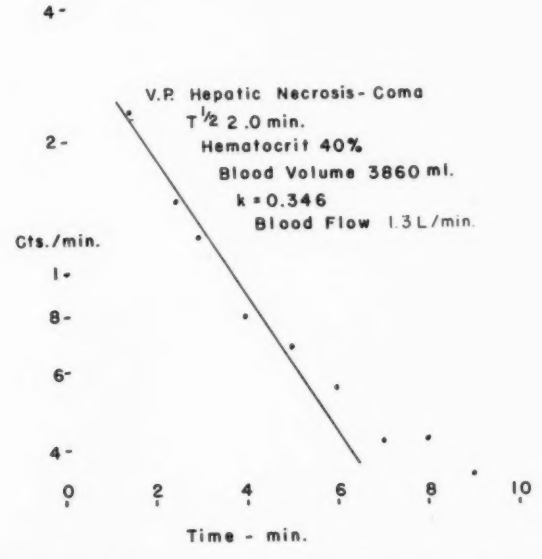

Fig. 3—This disappearance slope is within normal limits. The total counting rate was somewhat higher than that seen in normal cases. This man died with overwhelming hepatic necrosis 3 hours after completion of the study. Disappearance rate does not correlate in this patient with over-all hepatic function.

Congestive Heart Failure (Table V).—Disappearance rates in 7 patients with congestive heart failure are listed in Table V. The cases are listed roughly in order of the severity of their cardiac failure. All save the first man were bedridden with severe dyspnea, orthopnea, and edema. All of them had been completely digitalized. In these patients, in whom decreased hepatic perfusion was expected on both the strength of Myers'[13] previous report and a priori reasoning, the disappearance rates are slower than in normal individuals. The mean $T\frac{1}{2}$ was 2.5 minutes and k, 0.29. Mean blood flow was 1.3 L. per minute. The difference is not statistically significant. The BSP test has shown marked abnormality in the presence of congestive heart failure.[16] Unfortunately, standard BSP tests were not done on these patients simultaneously with the radiocolloid disappearance rates. From these data one can infer that the colloid is not presented to the removal site as rapidly in patients with congestive heart failure as it is in normal individuals.

Miscellaneous.—Small numbers of patients with a wide variety of diseases of the hepatobiliary system were studied (Table VI). Disappearance half times varied, with one exception (Case 6, a man with a gangrenous gall bladder), from 2.2 to 1.2 minutes—the range of the normal patients. This group of cases covers a wide variety of conditions and varied from ambulatory patients in the hospital

TABLE V. CHROMIC PHOSPHATE DISAPPEARANCE RATES IN CONGESTIVE HEART FAILURE

PATIENT NO.	AGE	SEX	BODY WEIGHT (KILOGRAMS)	CORRECTED HEMATOCRIT (%)	BLOOD VOLUME (ML.)	T½ (MINUTES)	k	HEPATIC BLOOD FLOW (L./MINUTE)	DIAGNOSIS
1	72	M	90.8	43.1	4,860	2.4	0.29	1.4	ASHD—mild
2	54	F	51.7	39.2		2.1	0.33		HASHD—moderate
3	70	F	49.4	36.0	4,080	3.6	0.19	0.8	ASHD—severe
4	72	M	90.8	47.7	4,230	2.0	0.35	1.5	ASHD—terminal
5	52	M	43.9	30.9	6,140	2.8	0.25	1.5	Syphilis—terminal
6	22	F	63.5	28.8	5,620	2.7	0.26	1.4	Unknown—very severe
7	63	M	51.7			2.0	0.35		Cor pulmonale—terminal
					Mean	2.5	0.29	1.3	

for study to desperately ill people, 3 of whom died within 48 hours of the study. From these data it is difficult to draw any conclusions about the effect of these disease syndromes on colloid disappearance rates.

DISCUSSION

European investigators[4, 5] have clearly established that colloidal gold disappearance rates, when treated mathematically as suggested by Dobson and Jones,[1] result in a figure for hepatic blood flow which agrees closely with that obtained by a number of authors[7] with the BSP method of Bradley.[2] Moreover, it has been established that the disappearance rate is slowed in cirrhosis of the liver and calculated hepatic blood flow is therefore less.[4, 5] The same result has been found with the use of the BSP removal method.[12]

The rate of removal of a colloid must depend on three factors: (1) the rate it is presented to the removal site in the liver or hepatic sinusoidal perfusion volume, which is not necessarily the same as the amount of blood flowing from the hepatic veins. For instance, in shock it has been demonstrated that there is decreased hepatic perfusion and decreased colloidal disappearance rates;[14] (2) the ability of the cells which remove the colloid to extract it completely from the blood perfusing these cells. Brauer[15] has shown that the chilled liver does not extract chromic phosphate as efficiently as does a warm liver; and (3) the rate of removal by extrahepatic sites of removal both in normal individuals and in severe hepatic disease. This is largely an unexplored concept.

The first of these variables is the one which has received almost exclusive attention from those authors who have worked with radioactive colloids. Yet, as one looks at the tabulated data, the choice of colloid seems to have as much to do with the results reported as does anything else. This stands in some distinction to the remarkable uniformity of results obtained with the BSP method.[7] The data reported herein suggest that chromic phosphate disappearance rates do not vary greatly in a wide variety of individuals severely ill with hepatobiliary disease. It is undoubtedly true that if flowing blood does not present a colloid to the hepatic cells, these cells cannot remove it from blood. However, little is known of what changes take place in phagocytic cell function in other parts of the body as hepatic disease progresses. Also, not much is understood of the phagocytic cell function in the liver itself in so-called hepatocellular disease. The presence of normal disappearance rates in individuals with severe jaundice forces one to consider the possibility that failing phagocytic cell function is not necessarily paralleled by failing Kupffer cell function. Cases 4 and 5 in Table VI were deeply jaundiced comatose individuals with homologous serum hepatitis. One died approximately 3 hours after the test was completed (Fig. 3). The second survived, but remains a chronic invalid. The normal disappearance rates (Fig. 3) in these persons in the presence of such impaired hepatic function suggests either that the Kupffer cells in the liver were functioning normally or that reticuloendothelial cells elsewhere in the body were effectively clearing the blood of the colloid. It is suggested that studies of colloidal behavior may be a more useful tool for investigating function of the reticuloendothelial system than for estimating hepatic blood flow.

TABLE VI. CHROMIC PHOSPHATE DISAPPEARANCE RATES IN MISCELLANEOUS CLINICAL CONDITIONS

PATIENT NO.	AGE	SEX	BODY WEIGHT (KILOGRAMS)	CORRECTED HEMATOCRIT (%)	BLOOD VOLUME (ML.)	T½ (MINUTES)	k	HEPATIC BLOOD FLOW (L./MINUTE)	DIAGNOSIS
1	39	F	59.0	27.4	5,220	2.0	0.35	1.7	Chromic hepatitis
2	51	F	51.0	18.0	3,270	1.4	0.49	1.6	Hepatitis—uremia
3	66	M		18.3	4,140	1.6	0.43	1.8	Hepatitis—uremia
4	62	F	57.0	32.0	4,170	1.4	0.49	2.1	Hepatitis—liver failure
5	55	M		40.0	3,860	2.0	0.35	1.3	Hepatitis—coma
6	65	M	77.3	27.6	5,950	3.0	0.23	1.4	Obstructive jaundice
7	47	F	61.3	28.8	4,870	1.5	0.46	2.6	Obstructive jaundice
8	59	F	58.6	38.7	3,810	1.8	0.39	1.5	Obstructive jaundice
9	48	M	49.5	26.1	5,450	1.6	0.43	2.4	Metastases to liver; ascites
10	69	M	66.4	41.4	6,920	1.6	0.43	3.0	Metastases to liver; ascites
11	36	F		38.7	4,760	1.2	0.58	2.8	Lymphosarcoma of liver; varices
12	24	M	62.8	31.7	3,440	1.8	0.39	1.3	Extrahepatic portal block
13	49	M	54.5	36.0	3,980	1.5	0.46	1.8	Thorotrast in liver
14	40	M	49.2	13.5	4,510	2.2	0.32	1.4	Sickle cell disease; jaundice

The most likely probability to explain the very rapid rate of disappearance of the colloid is extrahepatic removal or nonuniform mixture of the colloid in the blood of these very ill individuals at the time the first sample was taken.

CONCLUSIONS

1. Radioactive chromic phosphate disappearance rates in normal individuals allow estimation of hepatic blood flow.

2. In the presence of severe hepatocellular disease, radioactive colloid disappearance rates may be unchanged.

3. Other variables are involved in determining colloid disappearance rates which might invalidate their use for studying hepatic perfusion in clinical hepatocellular disease.

Addendum.—Subsequent to the completion of this paper, Warner and Vautrin (Clin. Res. 9:65, 1961) reported in abstract very little increase of T½ in subjects with liver disease (2.3 minutes) as compared to results obtained with normal subjects (2.9 minutes). Half-life remained low as compared to indocyanine green disappearance. Thus, they also report little change of chromic phosphate disappearance rates in severe hepatic disease.

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DIMINISHED ANTIDIURETIC RESPONSE TO PITRESSIN IN DIABETES INSIPIDUS DURING THE INFUSION OF SODIUM BICARBONATE SOLUTION

J. W. Czaczkes, M.D., Ph.D., M. Eliakim, M.D., and T. D. Ullmann, M.D.,
Jerusalem, Israel

The antidiuresis following injections of Pitressin shows considerable variations under various clinical and experimental conditions. In order to discover whether the urinary ionic composition is one of the factors responsible for these variations, the effect of exogenous Pitressin was compared during the infusion of sodium chloride and of sodium bicarbonate solutions in 3 patients suffering from diabetes insipidus of the pituitary type. In all 3 patients it was found that the antidiuretic effect of small doses of Pitressin was markedly less during the infusion of sodium bicarbonate as compared with that of sodium chloride solution. This difference may be due to a diminished sensitivity of the tubular membranes to Pitressin during the formation of alkaline urine. A parallel is drawn to the "Pitressin-resistant polyuria" in certain clinical and experimental conditions, in which the high urinary pH and other abnormalities of the urinary ionic composition may diminish the tubular responsiveness to exogenous and endogenous antidiuretic hormone.

DURING previous studies in this laboratory it was observed that injections of Pitressin* occasionally failed to produce maximal antidiuresis in normal subjects.[1] As this usually occurred after sodium bicarbonate had been given in order to render the urine alkaline, it was surmised that the antidiuretic effect of Pitressin might be modified by the pH and the ionic composition of the urine.

The following is a report on the antidiuretic response to small doses of Pitressin in 3 patients with diabetes insipidus during the intravenous administration of sodium chloride and of sodium bicarbonate solutions.

MATERIALS AND METHODS

In all 3 patients the diagnosis of diabetes insipidus of the pituitary type was made on the basis of their history, a positive result of the Hickey-Hare test, and a satisfactory clinical response to injections of Pitressin. The relevant clinical data and biochemical findings are summarized in Table I.

Experimental Procedure.—The patients were examined in the morning, no restriction on the intake of fluid having been imposed during the preceding night. A multi-eyed, soft rubber catheter was placed into the bladder and urine was collected every 10 minutes throughout the experiments. Blood samples were taken from the femoral artery by an

From the Departments of Medicine, A and B, and the Laboratory of Clinical Research, Rothschild-Hadassah University Hospital and the Hebrew University–Hadassah Medical School, Jerusalem, Israel.

Supported by a grant from the Hadassah Medical Organization Research Fund.
Received for publication June 30, 1960.

*Pitressin, L749B and J1539F, Parke, Davis & Company, Detroit, Mich.

indwelling needle. Urine and plasma were examined for total osmolarity by a thermistor type freezing point apparatus. Glomerular filtration rate was determined by the clearance of inulin.

Isotonic solutions of NaCl and of $NaHCO_3$ were given in various sequences, at a rate of 8.8 ml. per minute for the 2 adult patients (P. R. and H. R.) and 7.4 ml. per minute in the younger patient (N. S.). After urine flow and urinary osmolarity had become constant for at least 3 collection periods, one intravenous injection of Pitressin was given in amounts varying from 15 to 90 mu. After each injection urine flow and osmolarity were allowed to return to control levels for at least 3 collection periods before another injection was given or the solution was changed.

TABLE I. CLINICAL AND BIOCHEMICAL DATA OF 3 PATIENTS WITH DIABETES INSIPIDUS

			URINE		SERUM (MEQ./L.)			
PATIENT	AGE IN YEARS	SEX	DAILY OUTPUT (LITERS)	OSMOLARITY (MOSM./L.)	Na	K	Cl	OSMOLARITY (MOSM./L.)
P. R.	31	F	12.5-16	45	137-145	4.0-4.8	100-109	310-320
N. S.	14	M	7.5- 9	103-111	140-150	3.5-4.5	98-111	307
H. R.	21	M	18	60- 80	137	4	103-110	300

TABLE II. RESPONSE TO PITRESSIN INJECTIONS DURING THE INFUSION OF ISOTONIC SOLUTIONS OF SODIUM CHLORIDE AND OF SODIUM BICARBONATE IN DIABETES INSIPIDUS (PATIENT P. R.)

PERIODS (10 MINUTES)	V (ML./MINUTE)	U_{OSM} (MOSM./L.)	$U_{OSM} \times V$ MOSM/MINUTE	C_{H_2O} (ML./MINUTE)	G.F.R. (ML./MINUTE)	pH
0.85 per cent NaCl solution I.V. at 8.8 ml. per minute.—						
1-4	13.6	50	.670	11.4	92	6.0
30 mu. Pitressin I.V.—						
5	5.4	96	.520	3.7	63	6.0
6	1.4	373	.520	−0.3	74	6.0
7	2.0	393	.790	−0.6	100	6.0
8	6.0	156	.940	2.9	100	6.0
9	12.0	86	1.030	8.5	108	6.0
10	13.6	66	.900	10.6	89	6.0
11	16.0	66	1.060	12.4	106	6.0
1.26 per cent $NaHCO_3$ solution I.V. at 8.8 ml. per minute.—						
12-16	20	60	1.210	16.0	95	6.5-7.5
30 mu. Pitressin I.V.—						
17	22.0	66	1.460	17.1	103	7.5
18	18.0	70	1.260	13.7	83	7.5
19	18.0	70	1.260	13.7	85	7.5
20	18.0	70	1.260	13.7	83	7.5
21-23	22.0	70	1.560	16.7	99	7.5
90 mu. Pitressin I.V.—						
24	8.0	94	.750	5.5	54	7.5
25	5.0	220	1.100	1.3	87	7.5
26	9.4	165	1.550	4.2	124	7.5
27	15.8	106	1.630	10.3	116	7.5
28	22.0	66	1.460	17.1	95	7.5
29	22.0	86	1.890	15.6	113	7.5

V = urinary volume; U_{osm} = urinary osmolar concentration; $U_{osm} \times V$ = total solute excretion; C_{H_2O} = free water clearance; G.F.R. = glomerular filtration rate.

RESULTS

The results are illustrated in Figs. 1 through 3 and numerical data are presented in Tables II through IV. Positive values for C_{H_2O} are obtained when

the urine is hypotonic to plasma. Conversely, when water is reabsorbed by the renal tubules so as to make the urine hypertonic to plasma, this value becomes negative.

In the first 2 patients (P. R. and N. S.) the sodium chloride solution was infused first. In these patients the injection of 30 and 20 mu. of Pitressin, respectively, had an immediate and marked antidiuretic effect which lasted for about 40 minutes. When the same amounts of Pitressin were injected during the subsequent infusion of sodium bicarbonate there was only a slight antidiuretic effect in the first patient and no effect at all in the second patient. A triple dose of Pitressin, i.e., 90 and 60 mu., respectively, caused an antidiuretic response in both patients which, however, was less marked than the effect observed with the smaller doses of Pitressin given during the previous infusion of sodium chloride (Figs. 1 and 2).

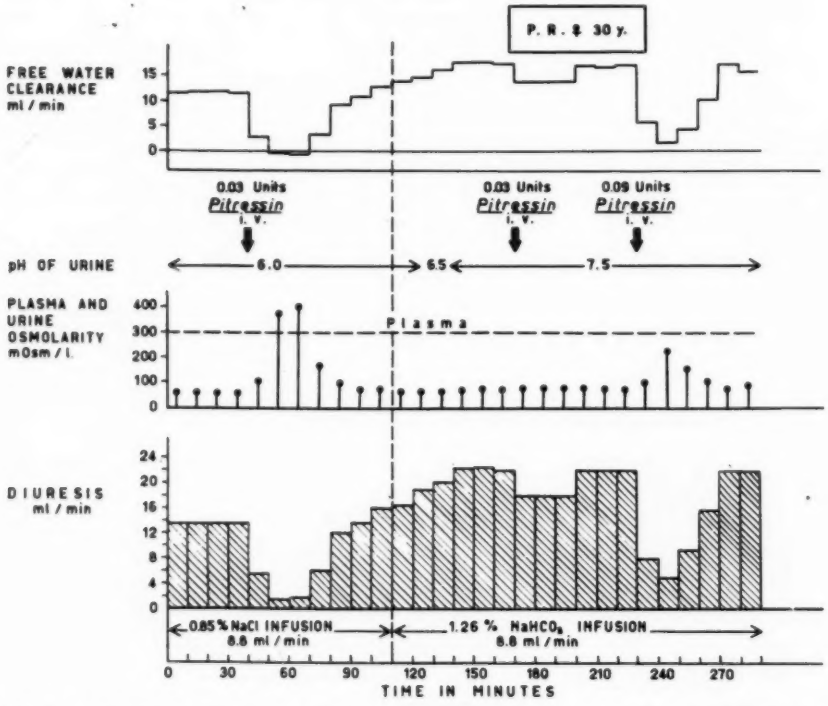

Fig. 1.—Changes in urine flow, urinary osmolarity, and free water clearance in Patient P. R. after Pitressin injection during the infusion of isotonic sodium chloride and sodium bicarbonate solution.

In the third patient (H. R.) the experiment was started with the infusion of the sodium bicarbonate solution. The injection of 30 mu. of Pitressin had a marked antidiuretic effect. Therefore, during the following infusion of sodium chloride solution, only 15 mu. of Pitressin was injected. This smaller amount resulted in antidiuresis definitely more pronounced and prolonged than that obtained with the double amount of Pitressin given previously during the sodium bicarbonate infusion (Fig. 3).

TABLE III. RESPONSE TO PITRESSIN INJECTIONS DURING THE INFUSION OF ISOTONIC SOLUTIONS OF SODIUM CHLORIDE AND OF SODIUM BICARBONATE IN DIABETES INSIPIDUS (PATIENT N. S.)

PERIODS (10 MINUTES)	V (ML./ MINUTE)	U_{OSM} (MOSM./L.)	$U_{OSM} \times V$ (MOSM./ MINUTE)	C_{H_2O} (ML./ MINUTE)	G.F.R. (ML./ MINUTE)	pH
0.85 per cent NaCl solution I.V. at 7.4 ml. per minute.—						
1-4	13.4	112	1.500	8.4	205	6.0
20 mu. Pitressin I.V.—						
5	6.0	111	.670	3.8		6.0
6	4.0	143	.570	2.1		6.0
7	1.5	266	.400	0.16	93	6.0
8	3.0	525	1.580	−2.3	148	6.0
9	8.0	410	3.300	−3.1		6.0
10	13.3	190	2.530	4.75	116	6.0
11	13.3	111	1.480	8.3		6.0
1.26 per cent $NaHCO_3$ solution I.V. at 7.4 ml. per minute.—						
12-14	13.4	113	1.510	8.25	175	6.5-8.0
20 mu. Pitressin I.V.—						
15-17	13.3	111	1.480	8.30	236	8.0
60 mu. Pitressin I.V.—						
18	10.0	130	1.300	5.55		8.0
19	5.0	200	1.000	1.56	144	8.0
20	6.0	143	.860	3.04	104	8.0
21	7.0	133	.930	3.8		8.0
22-23	13.3	111	1.480	8.2	154	8.0

V = urinary volume; U_{osm} = urinary osmolar concentration; $U_{osm} \times V$ = total solute excretion; C_{H_2O} = free water clearance; G.F.R. = glomerular filtration rate.

TABLE IV. RESPONSE TO PITRESSIN INJECTIONS DURING THE INFUSION OF ISOTONIC SOLUTIONS OF SODIUM BICARBONATE AND OF SODIUM CHLORIDE IN DIABETES INSIPIDUS (PATIENT H. R.)

PERIODS (10 MINUTES)	V (ML./ MINUTE)	U_{OSM} (MOSM./L.)	$U_{OSM} \times V$ (MOSM./ MINUTE)	C_{H_2O} (ML./ MINUTE)	G.F.R. (ML./ MINUTE)	pH
1.26 per cent $NaHCO_3$ solution I.V. at 8.8 ml. per minute.—						
1-4	13.8	56	.767	11.2	137	7.0
30 mu. Pitressin I.V.—						
5	7.2	100	.720	4.8	87	7.1
6	3.8	310	1.180	−0.17	126	8.0
7	7.2	123	.885	4.2	106	7.3
8	8.0	113	.905	4.9		7.3
9	10.0	83	.830	7.2		7.3
10	13.6	60	.815	10.8	125	7.3
0.85 per cent NaCl solution I.V. at 8.8 ml. per minute.—						
11-13	14.6	52	.750	12.0		7.0
15 mu. Pitressin I.V.—						
14	15.0	53	.795	12.3	126	7.0
15	3.8	123	.468	2.2	80	7.0
16	1.8	353	.638	−0.37	137	7.8
17	3.4	280	.950	0.15	177	7.6
18	9.2	93	.855	6.3	140	7.2
19	13.0	56	.725	10.5	135	6.8

V = urinary volume; U_{osm} = urinary osmolar concentration; $U_{osm} \times V$ = total solute excretion; C_{H_2O} = free water clearance; G.F.R. = glomerular filtration rate.

The changes in solute excretion ($U_{osm} \times V$) after the injection of Pitressin were parallel to those of the urinary output (Tables I through III). However, in some instances the total solute output, which had been initially reduced after the injection of Pitressin, together with the urinary flow, recovered more rapidly than the urine flow and even exceeded the control values. These peaks in solute excretion occurred while the urine flow was still markedly depressed, thus causing a more prolonged reduction in free water clearance than in urine flow. Such responses of solute excretion to Pitressin were observed during the administration of sodium chloride solution (Patient N. S., Periods 9 to 10) as well as during that of sodium bicarbonate solution (Patient R. H., Period 6).

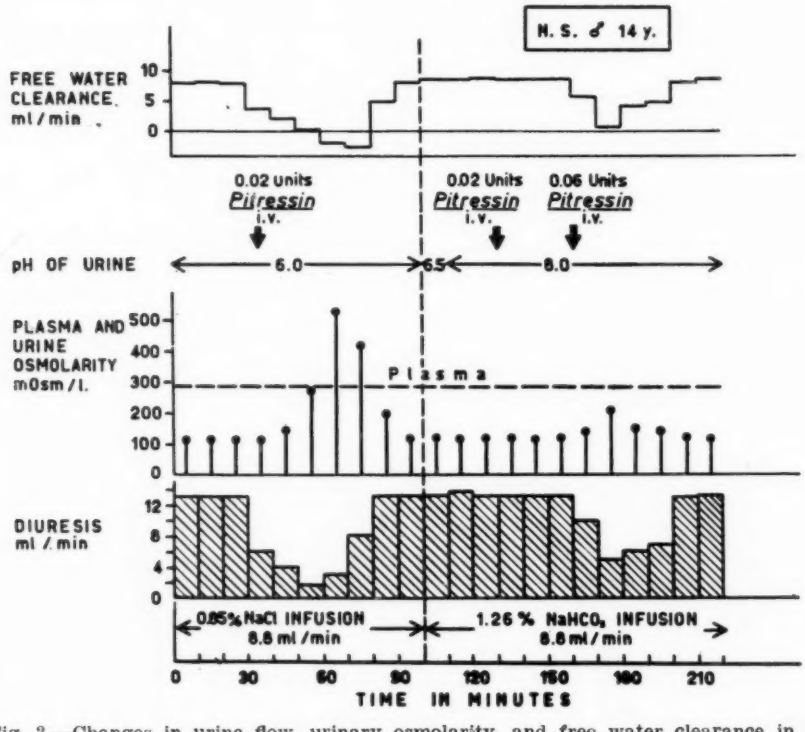

Fig. 2.—Changes in urine flow, urinary osmolarity, and free water clearance in Patient N. S. after Pitressin injections during the infusion of isotonic sodium chloride and sodium bicarbonate solutions.

The glomerular filtration rate was temporarily lowered after the injection of Pitressin and urine flow and osmolar output were decreased. The subsequent rises back to and above control values were more closely parallel to the rise in osmolar output than to the increase in urine flow.

The changing of the solutions from sodium chloride to sodium bicarbonate in Patients P. R. and N. S. caused a rise in urinary pH from 6 to 7.5 and 8, respectively. No further changes were observed following the injection of Pitressin. In the experiment on Patient R. H., in which the sodium bicarbonate solution was infused first, the pH was about 7 throughout; a rise to 8 (7.9), after the injections of Pitressin, occurred together with the increased solute excretion.

DISCUSSION

The antidiuretic response to small amounts of Pitressin in patients with diabetes insipidus of the pituitary type was found to be less during the infusion of sodium bicarbonate solution than during the infusion of sodium chloride solution. This difference cannot be ascribed to a mechanism of "solute diuresis," as the osmotic load was kept constant throughout the experiments and the urine specimens were markedly hypotonic to plasma before the Pitressin injections were given. It can therefore be concluded from our observations that during the administration of sodium bicarbonate solution the tubular cells did not react to small doses of Pitressin in the same way as they did during the administration of sodium chloride.

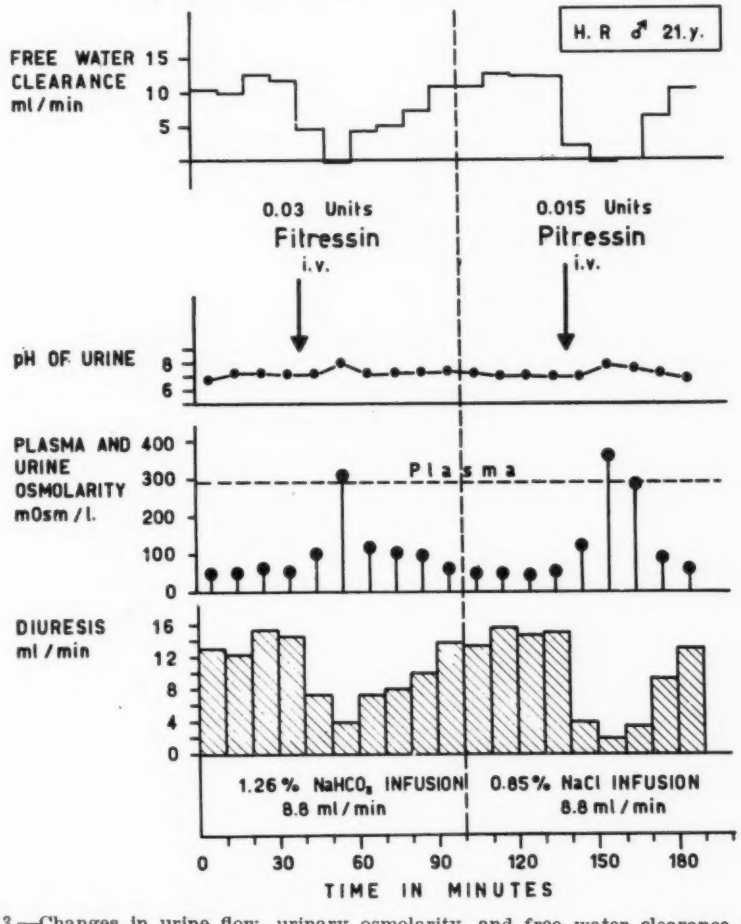

Fig. 3.—Changes in urine flow, urinary osmolarity, and free water clearance in Patient H. R. after Pitressin injections during the infusion of isotonic sodium bicarbonate and sodium chloride solutions.

In a recent study, Alexander and associates[2] found that patients (as well as experimental animals) with diabetes insipidus excreted urine of decreased hydrogen ion concentration, and that vasopressin did not raise the urinary concentration to normal values. In the light of our observations it appears

possible that the failure to produce highly concentrated urine in the presence of exogenous Pitressin may be connected with the high urinary pH of the patients with diabetes insipidus.

In this connection, it may be recalled that alkaline urine specimens, examined at random, have a significantly lower specific gravity and are excreted in larger daily volumes than specimens of acid urine.[3] These observations may perhaps also be explained by the assumption that the renal tubules are less sensitive to endogenous Pitressin during the excretion of alkaline urine.

A "Pitressin-resistant polyuria" has been described in a number of clinical and experimental conditions: in hyperparathyroidism,[4] during the administration of parathyroid extract,[5] in hypercalcemic states,[6-9] in some cases of hyperthyroidism,[10] during the administration of desoxycorticosterone acetate,[11, 12] in primary aldosteronism,[13, 14] and in hypokalemic states of other origin.[15-17] Urinary alkalinity and other abnormalities of the urinary ionic composition are common in these conditions.[4, 8, 9, 13, 14, 18, 19] Abnormalities in the composition of the interstitial fluid of the renal papillae recently have been described for some of them.[20, 21] It appears that the resistance to Pitressin in these conditions may be caused by the surplus, or lack, in the tubular urine or in the interstitial fluids, of certain ions, which are critical for the responsiveness to Pitressin of the renal tubules and collecting ducts.

In a similar way, the diminished sensitivity to Pitressin during the administration of sodium bicarbonate solution in patients with diabetes insipidus may be caused by the alkalinity of the urine, or by related changes in its ionic composition. Further studies are needed to decide which of the abnormalities of the ionic structure of the tubular urine and the interstitial fluid interfere with the antidiuretic action of Pitressin.

SUMMARY

Isotonic sodium chloride and sodium bicarbonate solutions were given intravenously to 3 patients with diabetes insipidus of the pituitary type. Pitressin injections during these infusions were found to be less effective in causing antidiuresis during the infusion of sodium bicarbonate solutions than during the infusion of those of sodium chloride. This difference is ascribed to a diminished sensitivity of the tubular cells to Pitressin during the excretion of alkaline urine. A parallel is drawn to the "Pitressin-resistant polyuria" found in certain clinical conditions, in which the high urinary pH and other abnormalities of the urinary ionic composition may diminish to tubular responsiveness to endogenous and exogenous Pitressin.

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SERUM ENZYMES IN DISEASE

VI. GLUTATHIONE REDUCTASE IN SERA OF NORMAL SUBJECTS AND OF PATIENTS WITH VARIOUS DISEASES

MICHAEL WEST, M.D., CYRENA BERGER, M.D., HUGO RONY, M.D., AND HYMAN J. ZIMMERMAN, M.D.
CHICAGO, ILL.

Serum glutathione reductase has been determined in approximately 800 patients with various diseases and in 124 normal subjects. Children have a slightly higher mean (60 units) than do adults (43 units). Moderate elevations of glutathione reductase in some patients were found in all the major categories of disease studied.

The greatest incidence of abnormal levels were found in patients with carcinoma, leukemia, hepatitis, cirrhosis, megaloblastic anemia, and hemoglobin S-S disease.

It is suggested that elevations of this enzyme in the serum may in part be related to its extramitochondrial location in the cell as well as to diseases affecting the cell.

THAT glutathione reductase (GR) is an enzyme which, in the presence of the co-enzyme triphosphopyridine nucleotide, can catalyze the conversion of oxidized glutathione to reduced glutathione, has been demonstrated in the serum of animals[1] and humans.[1, 2] Manso and Wroblewski[1] have reported consistent elevations of this enzyme in the serum of patients with viral and toxic hepatitis and (usually) disseminated carcinoma. Kerppola, Nikkila, and Pitkanen[2] reported similar findings in a larger series of 365 patients. The present report deals with a study of the serum level of this enzyme in normal subjects and 798 patients with neoplastic, hepatic, cardiac, and other diseases.

MATERIALS AND METHODS

The principle of the analysis for glutathione reductase (GR) activity is the quantitative oxidation of reduced triphosphopyridine nucleotide (TPNH) by serum in the presence of oxidized glutathione as substrate. The procedure of Racker,[3] with some modifications, was used in the present study. In performing the assay the following mixture is incubated at 37° C. for 20 minutes: 0.2 ml. serum, 2.6 ml. of 0.010 M phosphate buffer (pH 7.5), and 0.1 ml. of 0.002 M TPNH solution. The reaction is initiated by the addition of 0.1 ml. of 0.033 M oxidized glutathione. The change in optical density of the reaction mixture, using a blank from which TPNH has been omitted, is recorded at 340 mμ, at 30 second intervals,

From the Department of Medicine, The Chicago Medical School, The Mount Sinai Hospital, and The Park View Home for the Aged, Chicago, Ill.

Supported by United States Public Health Service Grant (No. C-4321), an American Cancer Society Grant (P-217), and the Chicago Heart Association.

Received for publication Sept. 23, 1960.

for 6 minutes, in a Beckman DU or DK spectrophotometer, the cells of which are maintained at 37° C. It is often necessary to dilute "active" sera to obtain "zero order" reaction kinetic curves.

The activity of GR in the serum is measured by the rate of disappearance of TPNH. Other authors[1, 2] have reported the activity of GR in the serum in units. Accordingly, the same unit of activity will be used in this report. A unit of activity is defined as a change in optical density at 340 mμ in the Beckman DU spectrophotometer of 0.001 per milliliter of serum per minute at 37° C. One such unit is equivalent to 0.48×10^{-3} μM of TPNH oxidized per milliliter of serum per minute at 37° C.

The solutions of oxidized glutathione* and reduced TPNH* solutions are prepared every day.

Samples acquired from subjects in the fasting state did not differ significantly from those obtained postprandially. Although plasma and serum do not differ significantly in their enzyme content, serum was used in all determinations. Hemolysis increases the serum levels, since the erythrocyte content of GR is 2 or 3 times that of plasma.[1] Specimens showing hemolysis were discarded. Storage of serum at −10° C. for 7 days or more did not reduce serum GR activity. Most determinations were performed within 4 hours after serum was obtained.

TABLE I. SERUM GLUTATHIONE REDUCTASE ACTIVITY IN NORMAL SUBJECTS AND IN PATIENTS WITH VARIOUS DISEASES

DIAGNOSIS	NO. OF PATIENTS	GR ACTIVITY (UNITS)			
		11 TO 75	76 TO 100	101 TO 200	> 200
Normal adult	60	60	0	0	0
Normal child	64	57	7	0	0
Neoplastic.—					
Benign	24	24	0	0	0
Malignant	212	135	35	31	11
Leukemia	25	10	4	7	4
Lymphoma	37	32	3	2	0
Multiple myeloma	2	2	0	0	0
Viral hepatitis	28	8	7	12	1
Cirrhosis	39	24	5	10	0
Infectious mononucleosis	11	5	3	3	0
Biliary tract disease	26	18	6	2	0
Myocardial infarction	81	66	10	5	0
Coronary insufficiency	34	28	4	2	0
Congestive heart failure	61	57	1	3	0
Myocarditis	4	4	0	0	0
Renal disease	14	11	1	2	0
Pulmonary disease	20	10	7	3	0
Megaloblastic anemia	4	0	1	3	0
S-S disease	4	0	0	4	0
Iron deficiency anemia	7	7	0	0	0
Miscellaneous	165	149	13	3	0
Total	922	707	107	92	16

RESULTS

Normal Subjects.—Sixty normal individuals ranging in age from 18 to 60 years were studied. The mean GR value for this group was 43 ± 16 units. The normal range accordingly, was considered to be 11 to 75 units. There was no significant difference between results obtained from the men and women in this group.

*These two substances were obtained from the Sigma Chemical Company, St. Louis, Mo., and used without further purification.

Sixty-four normal children, ranging in age from 4 months to 18 years, also were surveyed. The mean of this group was 60 ± 16 units, resulting in a range of 28 to 92 units. There did not appear to be any significant difference between those children below 3 years of age and those 3 to 18 years old.

Exclusive of the normal group, 798 patients with various diseases were studied. Approximately half of the patients had two or more determinations on different occasions. For as clear presentation of data as possible, only the highest value obtained in each patient was considered (Table I and Fig. 1).

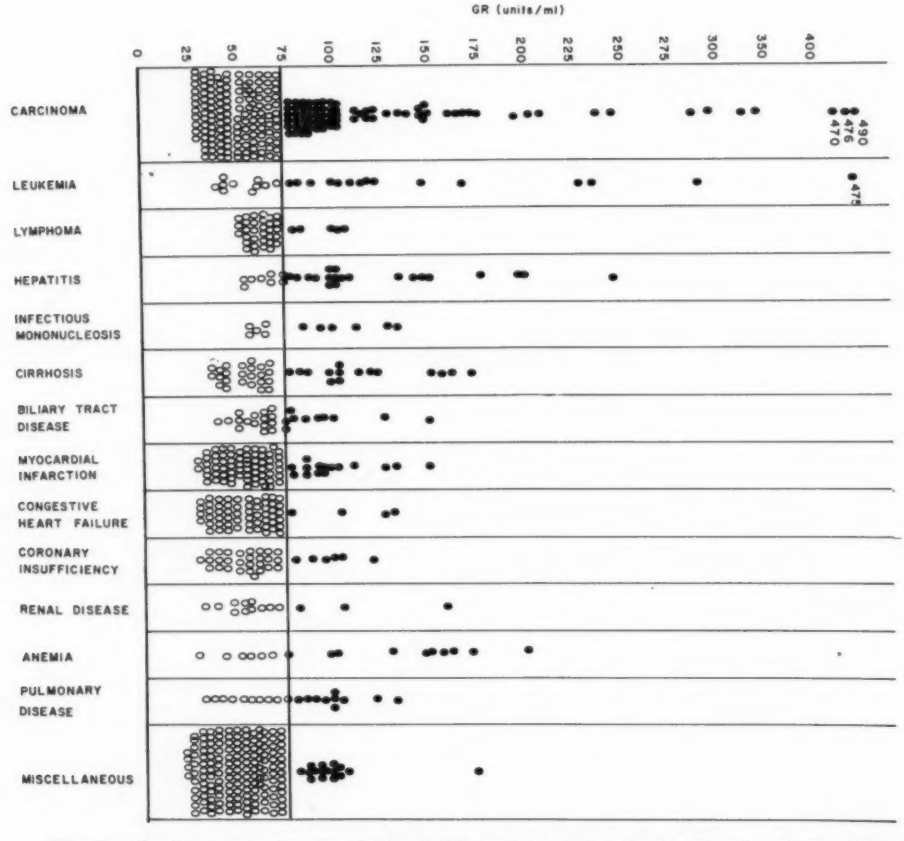

Fig. 1.—Scattergram showing highest GR values determined. Levels above 200 units were almost exclusively found in patients with carcinoma or leukemia. The black circles represent abnormal values, the white circles, normal values.

Neoplastic Disease.—There were 292 patients in this group. No serum GR elevations were found in the 24 patients considered to have benign tumors, including such entities as leiomyofibromas of the uterus, fibroadenomas of the breast, lipomas and fibromas of the skin, and pulmonary adenomas.

Fifty-four patients had various types of malignant tumors without apparent metastases (Table II). Twelve of these patients demonstrated moderate elevations (75 to 200 units).

The group with suspected or proved metastatic carcinoma consisted of 158 patients. Sixty-five of these patients had values over 75 and 9 had values over

200 units. Serum GR elevations were found in patients with metastatic carcinoma even where there was no evidence of liver or bone involvement. Nevertheless, the patients with these 2 sites of metastases had the highest incidence of elevations. This was particularly evident in patients with bone metastases (Table II).

TABLE II. RELATIONSHIP OF ABNORMAL GR LEVELS TO METASTATIC SITE

SITE OF METASTASES	NO. OF PATIENTS	NO. OF ABNORMAL	PER CENT ABNORMAL
None	54	12	22
Liver without bone	22	10	45
Liver with bone	13	8	61
Bone without liver	25	16	64
Others (lung, nodes, pleura, etc.)	98	33	33

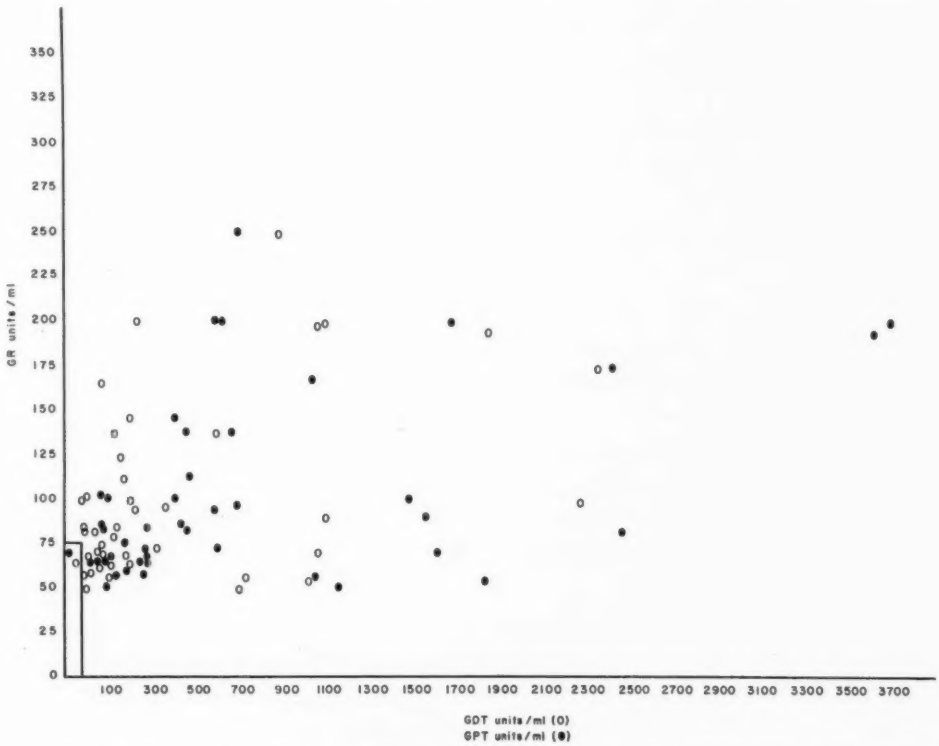

Fig. 2.—Scattergram showing GR, GOT, and GPT values simultaneously determined in patients with viral hepatitis. Elevated transaminase levels occurred frequently even when the GR levels were normal or slightly abnormal.

Fifteen of the 25 patients with leukemia had abnormal serum GR levels. Although this was a small group, it is of interest that patients with untreated acute leukemia and chronic granulocytic leukemia usually had elevated levels of GR while those with chronic lymphocytic leukemia had normal GR levels.

It is of interest that GR values above 200 units were found almost exclusively in patients with carcinoma or leukemia. Only one patient without neoplastic disease (viral hepatitis) had a value above 200 units.

Only five of the 37 patients with various forms of malignant lymphoma had abnormal levels and these were all below 125 units. The 2 patients with multiple myeloma had normal GR levels.

Hepatic and Biliary Tract Disease.—Determinations were performed in 67 patients in this group. Twenty of the 28 patients with viral hepatitis demonstrated elevations; one patient had a value over 200 units (242 units). Serum GR levels were usually elevated in the first week of illness. Nevertheless, several patients had normal or only slightly elevated levels when the transaminases (GOT and GPT) were elevated to 60 times the normal. Fig. 2 is a scattergram plotting all the GR, GOT, and GPT values determined in the patients with hepatitis. One patient with "granulomatous hepatitis" due to tuberculosis had a value of 120 units.

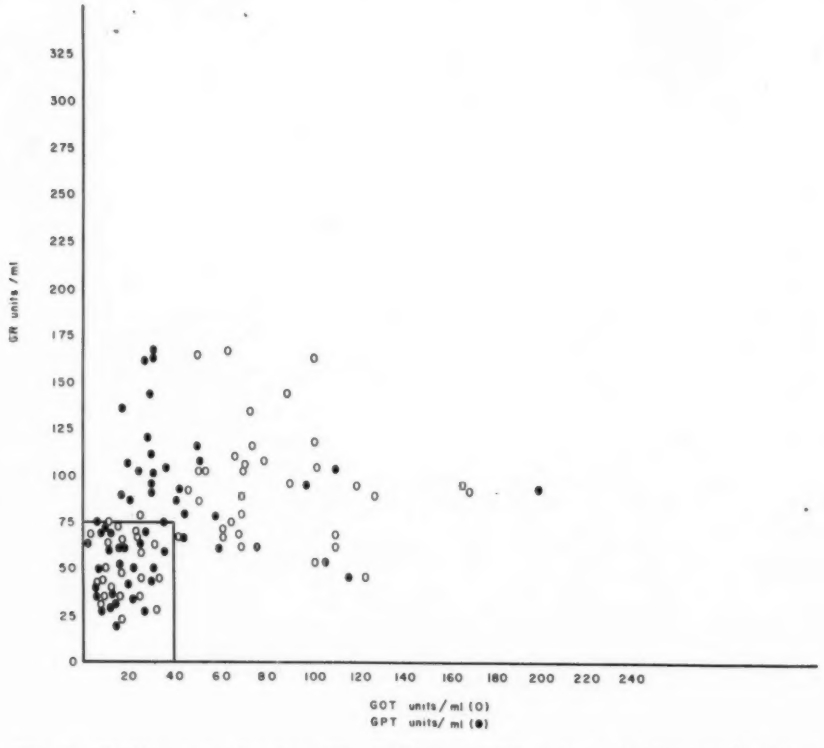

Fig. 3.—Scattergram showing GR, GOT, and GPT values simultaneously determined in patients with cirrhosis of the liver. Elevated GR values were observed in patients with normal GPT values, while abnormal GOT or GPT values were found in patients with normal GR levels.

Of the 39 patients with cirrhosis of the liver (Laënnec's, postnecrotic, and biliary), 15 had moderate elevations, none exceeding 180 units. Ten patients had normal GR levels with elevated GOT levels, while only one patient had normal GOT and elevated GR levels (78 units) (Fig. 3). Elevations of GR in patients with cirrhosis did not appear to be related to such associated findings as jaundice, ascites, or coma.

There were 26 patients with extrahepatic biliary tract disease such as common bile duct obstruction or acute cholecystitis. Only 8 of these patients had elevated GR values, even though the majority of patients in this group had elevated transaminase levels and were jaundiced.

Mild GR elevations were found in 6 of 11 patients with infectious mononucleosis. All of these patients had abnormal transaminase levels as evidence of hepatic involvement.

Cardiac Disease.—There were a total of 180 patients in this group. Eighty-one patients with acute myocardial infarction were studied at least once within 48 hours of the time of the infarction. Fifteen patients had abnormal levels, the highest being 150 units. No elevations occurred after 48 hours. In some patients, elevations were present on the second day but not on the first.

It is of interest that 6 out of a group of 34 patients with symptomatic coronary insufficiency but without evidence of acute myocardial infarction, congestive heart failure, or hypotension had mild elevations of GR, none exceeding 125 units.

Sixty-one patients with congestive heart failure were studied. Only four had abnormal levels (85, 105, 125, and 130 units). All of these patients had predominately left heart failure.

Three patients with acute rheumatic myocarditis and one with a viral myocarditis had normal levels.

Anemias.—Four patients with megaloblastic anemia in relapse had elevated GR levels (90 to 200 units). Three of these had Addisonian pernicious anemia. The patient with megaloblastic anemia due to a malabsorption syndrome had the least elevated value (90 units).

Four patients with hemoglobin S-S disease also had elevated values (150, 150, 162, and 168 units). One patient with a hypoplastic anemia had a mildly elevated value of 100 units.

There were 7 patients with iron-deficiency anemia; all had normal GR levels.

Renal Disease.—There were 14 patients in this group with various types of acute and chronic renal diseases. Elevations were demonstrated in only 3 of these patients; one with malignant nephrosclerosis (157 units); another with nephrosclerosis (120 units); and the third with chronic pyelonephritis (80 units). Nine of the 14 patients had varying degrees of azotemia.

Pulmonary Disease.—Of nine patients with acute "pneumonitis," 4 had mild elevations (90, 98, 123, and 138 units). Five patients with chronic pulmonary disease were studied; 2 mild elevations were noted (85 and 80 units). Two patients with active pulmonary tuberculosis had normal levels, while one with sarcoidosis had a level of 110 units. One of 2 patients with pulmonary infarction had an elevated serum GR level (98 units), as did a patient with pleural effusion apparently associated with rheumatoid arthritis.

Miscellaneous.—There were a total of 165 patients in this category. In a group of 71 patients with various diseases such as duodenal ulcer with and without bleeding, gastroenteritis, esophagitis, hiatus hernia, fibrositis, influenza, and diabetes mellitus there were no elevated serum GR levels.

In another group of 74 aged patients (all over 65 years of age) with mainly degenerative diseases such as hypertensive heart disease, coronary heart disease, chronic bronchitis, Parkinson's disease, peripheral vascular disease, benign prostatic hypertrophy, osteoarthritis, emphysema, rheumatoid arthritis, diabetes mellitus, psoriasis, glaucoma, tertiary syphilis, obesity, and Paget's disease of the bone, there were 4 patients with slight elevations of serum GR levels, none above 100 units.

Two of 4 patients with pre-eclampsia had slightly elevated values (90 and 100 units, respectively). No patients with eclampsia were studied.

One patient with a subphrenic abscess had a GR value of 87 units, one with a pelvic abscess had a value of 170 units, and one with generalized furuncles had a value of 101 units.

Two of 3 patients with "collagen disease" (probably systemic lupus erythematosus) had values of 90 and 115 units.

One of 5 patients with thyrotoxicosis had a value of 99, while one out of 2 patients with myxedema had a GR level of 78 units.

Values of 80, 93, and 80, respectively, were found in 3 patients; one with diabetic coma, another with a cerebrovascular "accident," and the other with essential hypertension.

DISCUSSION

The exact role of glutathione reductase (GR) in tissue is not clear. It has been shown, however, that reduced glutathione either protects or activates certain sulfhydryl enzymes and proteins.[4] The metabolic role of glutathione reductase may be to provide adequate amounts of reduced glutathione. Schrier and his associates[5] have suggested that glutathione reductase acts in the electron transport chain for the hexose monophosphate shunt in the human erythrocyte. Even though the normal serum contains GR it contains no demonstrable reduced glutathione (GSH).[6] On the other hand, the presence of GSH in tissue cells and erythrocytes may be related to the associated presence of triphosphopyridine nucleotide (TPNH), which is not normally present in the serum. (Serum does not reduce oxidized glutathione [GSSG] unless TPNH is added.) Another fact which may explain the absence of GSH in serum may be related to the capacity of serum to oxidize GSH to GSSG.[7] Thus, the mechanisms which operate in the cell to maintain an adequate level of GSH are not present in the serum.

No relationship between the role of GR in tissue and the occurrence of elevated serum levels is apparent at present.

In this[1] and one other study[2] it has been shown that human serum always contains detectable activities of GR. The mean in adults was 43 units, in children, 60 units. There appeared to be no sex differences.

Glutathione reductase activity is highest in homogenates of human liver and kidney; pancreas, heart, thyroid, and lung have decreasing activity in that order.[1]

The observation of elevated levels of glutathione reductase in the serum of some patients with neoplastic disease supports the observations of Manso and Wroblewski[1] and of Kerppola, Nikkila, and Pitkanen.[2] The former investigators reported elevations only in patients with hepatitis and carcinoma in a series of 104 patients. Kerppola and his associates, in a study of 365 patients, reported elevations in some patients with cirrhosis, hepatitis, leukemia, pulmonary sarcoidosis, and carcinoma.

The results of our study of 798 patients reveal elevations in patients with a variety of diseases including hepatitis, cirrhosis, infectious mononucleosis, biliary tract disease, carcinoma, leukemia, lymphoma, myocardial infarction, congestive heart failure, coronary insufficiency, pernicious anemia, S-S disease, and various acute and chronic infectious diseases. The greatest incidence of elevations were seen in patients with carcinoma, leukemia, hepatitis, cirrhosis, pernicious anemia, and S-S disease.

In patients with various liver diseases, GR levels appear to be less consistently and less markedly elevated than those of the transaminases. The incidence and degree of elevation of GR in patients with hepatitis is not striking.

The incidence of elevations in patients with megaloblastic anemia and S-S disease is similar to those of serum lactic dehydrogenase.[8-10]

Of interest are the results obtained in patients with neoplastic disease. Approximately 20 per cent of patients without evident metastases had elevated GR levels, while the largest percentage of patients with elevations were those with bone involvement. Manso and his associates[11] have reported elevated plasma GR levels in animals with transplanted tumors, apparently without metastases. Further studies are being done in this laboratory in an attempt to correlate GR elevations in patients with carcinoma, with the type of primary lesion, presence or absence of metastases, and their site.

Patients with untreated acute leukemia or chronic myelogenous leukemia had significant elevations, while those with these two types of leukemia in remission and patients with chronic lymphocytic leukemia had normal values. Elevations in patients with the various entities comprising the malignant lymphoma group were infrequent. The results in patients with leukemia and malignant lymphoma are quite similar to results obtained with serum lactic dehydrogenase studies.[12]

The basis for the elevated levels of this enzyme in the various diseases studied is obscure. Release from necrotic tissue may contribute to the elevated serum levels. In addition, glutathione reductase is a "soluble" enzyme, since it is found in the cytoplasm of cells,[13] and, therefore, slight changes in the permeability of the cell membranes may result in increased release into the serum.

Elevated serum levels of this enzyme are found in too many conditions to have specific diagnostic significance. The incidence and degree of abnormality in patients with neoplastic disease may find clinical applicability. Data from the present study suggest that GR values above 200 units may indicate the presence of carcinoma or leukemia.

SUMMARY

1. Serum glutathione reductase has been determined in 798 patients with various diseases and 124 normal control subjects.

2. Although there were moderate elevations of glutathione reductase in some of the patients in each major category of disease studied, the greatest incidence of abnormal levels was in patients with neoplastic, hepatic, and megaloblastic anemia, and hemoglobin S-S disease.

3. The explanation for serum elevations of this enzyme are not clear, although they may in part be related to the extramitochondrial location of the enzyme in the cell as well as to the diseases affecting the cell.

We wish to express our appreciation to Dorothy Gantt and Jerome Fifer for their technical assistance.

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LOSS OF I^{131} TAG FROM RADIOIODINATED 4-IODOANTIPYRINE AFTER ITS INJECTION IN THE RAT

JAY M. SULLIVAN, B.S.,* AND JOHN C. ROSE, M.D.
WASHINGTON, D. C.

At intervals following the intraperitoneal injection of radioiodinated 4-iodoantipyrine (4-I^{131} AP) in rats, samples of brain, muscle, and plasma were counted for radioactivity. Despite approximately equal water content of brain and muscle, significantly higher radioactivity was found in muscle than in brain at 1 and 2 hours after injection. No "blood-brain barrier" was found when samples were obtained earlier. The possibility was then tested that with time the I^{131} tag leaves the antipyrine molecule. Five groups of one hour comparative dialyses were performed: 4-I^{131} AP mixed with plasma in vitro; with water in vitro; and with rat plasma at 15, 60, and 120 minutes following intraperitoneal injection of 4-I^{131} AP. Data were expressed as per cent radioactivity remaining inside the cellophane membrane. Results were: I^{131} AP plus plasma in vitro, 50 per cent; I^{131} AP plus water, 30 per cent; I^{131} AP in plasma in vivo 15 minutes, 17 per cent; 60 minutes, 7 per cent; 120 minutes, 4 per cent. The first result is due to reversible protein binding. The in vivo results suggest that soon after injection of 4-I^{131} AP, significant and increasing quantities of the I^{131} tracer are detached from the antipyrine molecule in a form which is more diffusible than 4-I^{131} AP. Yet, this new form does not pass freely the blood-brain barrier. Evidence for a similar phenomenon is seen in other species. There are, therefore, qualitative differences in the behavior of antipyrine and 4-I^{131} AP when injected in animals, and these may invalidate the use of 4-I^{131} AP as a substitute for antipyrine in studies of body composition.

THE blood-brain barrier is freely permeable to water and to substances that distribute themselves in the total body water.[1] Radioiodinated 4-iodoantipyrine (4-I^{131} AP)† is considered to exhibit the same properties, such as volume of distribution and diffusibility, that antipyrine possesses, and thus has been used for the measurement of total body water in man,[2] and total body water[3] and cerebral blood flow[4] in animals. During the course of studies of the blood-brain barrier in rats, using 4-I^{131} AP as a reference for comparison with other substances, we have noted higher concentrations of radioactivity due to I^{131} in rat muscle than in rat brain. Yet the water content of these tissues is approximately equal.[5] The increasing use of this agent as a tool for

From the Department of Physiology and Biophysics, Georgetown University Schools of Medicine and Dentistry, Washington, D. C.

Received for publication Oct. 10, 1960.

*Work done during the successive tenures of Medical Student Research Fellowships from the Washington Heart Association and the United States Public Health Service.

†Abbott Laboratories, North Chicago, Ill.

the study of the circulation and body composition indicated that further studies on its behavior might be of significance in clarification of the extent of its usefulness.

The studies reported here are confined to the rat. First, the relative concentrations of 4-I^{131} AP in rat brain and rat muscle were studied at intervals following injection. Second, the diffusibility of radioactivity from rat plasma obtained at varying time intervals after injection of 4-I^{131} AP was investigated. The results indicate rapid breakdown of the 4-I^{131} AP molecule in this species so that, after a period, the measurement of radioactivity in tissue or fluid samples does not appear to be a measure of 4-I^{131} AP concentration.

TABLE I. RELATIVE CONCENTRATIONS OF I^{131} FROM 4-I^{131} AP IN RAT BRAIN AND MUSCLE, REFERRED TO THE PLASMA CONCENTRATION (STANDARD DEVIATIONS ARE GIVEN)

TIME AFTER INJECTION (MINUTES)	NO. OF RATS	BRAIN/PLASMA RATIO OF COUNTS	MUSCLE/PLASMA RATIO OF COUNTS
4-8	6	0.52 ± 0.12	0.28 ± 0.07
9-11	6	0.43 ± 0.09	0.36 ± 0.03
12-20	12	0.35 ± 0.06	0.42 ± 0.04
25-35	6	0.24 ± 0.05	0.35 ± 0.04
55-66	6	0.09 ± 0.02	0.23 ± 0.05
115-125	6	0.03 ± 0.01	0.18 ± 0.01

MATERIALS AND METHODS

Experiment I.—Apparent Concentration of 4-I^{131} AP in Brain Vs. Muscle.—Young adult male Sprague-Dawley rats which averaged 150 grams in weight were used. They were fed a normal diet ad libitum, and allowed free access to water until injection of 4-I^{131} AP. All rats were anesthetized with sodium pentobarbital (50 mg. per kilogram body weight) administered approximately 15 minutes before performance of cardiac puncture. Five microcuries of 4-I^{131} AP in 1 ml. of solution was injected intraperitoneally. Following injection, at stated intervals, cardiac puncture was performed with a heparinized syringe and a 22 gauge needle. The blood was centrifuged and 0.5 ml. plasma was pipetted into a counting tube.

Immediately after cardiac puncture, an incision was made over the right gastrocnemius muscle. A section approximately 1 cm. in length was excised, rapidly rinsed in distilled water for removal of adherent blood, blotted dry, weighed on a torsion balance, and placed in a counting tube with 4 ml. of distilled water.

A triangular section of skin was then removed from over the skull. The skull was opened with bilateral incisions, and the roof removed. A scoop was placed beneath the cerebellum, which severed the medulla oblongata and removed the remainder of the brain from the cranial cavity. This sample was then rinsed, dried, weighed, and placed in a counting tube in the same fashion as was the muscle.

All the samples were counted in a well-type scintillation counter, and radioactivity was converted to counts per minute per gram or milliliter. The ratios of radioactivity of brain and of muscle to plasma were then calculated.

Experiment II.—Diffusibility of Radioactivity Following Injection of 4-I^{131} AP.—Five groups of comparative dialyses were performed with a boiled cellophane membrane 80 μ thick (pore size 30 Å). Volumes of dialysant to distilled water dialysate were in the ratio of 1:20. In each instance, the cellophane tubing containing the radioactive dialysant was suspended in a beaker of distilled water and the dialysis was permitted to proceed for 1 hour. At the end of the dialysis period, volumes were measured and samples from both sides of the membrane were removed for counting. The control predialysis sample was counted at the same time and the per cent of radioactivity remaining inside the membrane at the end of dialysis was calculated. The 5 groups were composed as follows:

Group A: 4-I^{131} AP was mixed in vitro with plasma obtained by cardiac puncture of several young rats. The concentration was approximately 0.04 μc per milliliter plasma. The mixture was permitted to stand for 1 hour at room temperature. Six measured samples were dialyzed as described above.

Group B: 4-I^{131} AP was mixed in vitro with distilled water. The concentration was approximately 0.04 μc per milliliter water. Six measured samples were dialyzed as described.

Groups C, D, and E: 4-I^{131} AP was mixed in vivo in rat plasma. Each rat was injected with 10 μc 4-I^{131} AP in 1 ml. solution, intraperitoneally. At 15 minutes (Group C), 60 minutes (Group D), and 120 minutes (Group E), cardiac puncture was performed. The plasma obtained was dialyzed as described.

Two additional control experiments were performed. Rats pretreated with 4 drops Lugol's solution daily for 2 days were handled precisely as were the animals in Groups D and E. Six animals treated with Lugol's solution were injected for 1 hour, and 6 for 2 hours. Brain and muscle to plasma ratios, as well as plasma dialysis of radioactivity, were determined in these animals.

The second additional control experiment involved the use of additional "carrier" antipyrine. In 6 rats injected for 1 hour and 6 injected for 2 hours, the injected 4-I^{131} AP was accompanied by 2 mg. nonradioactive antipyrine. Plasma dialyses were performed in the manner described.

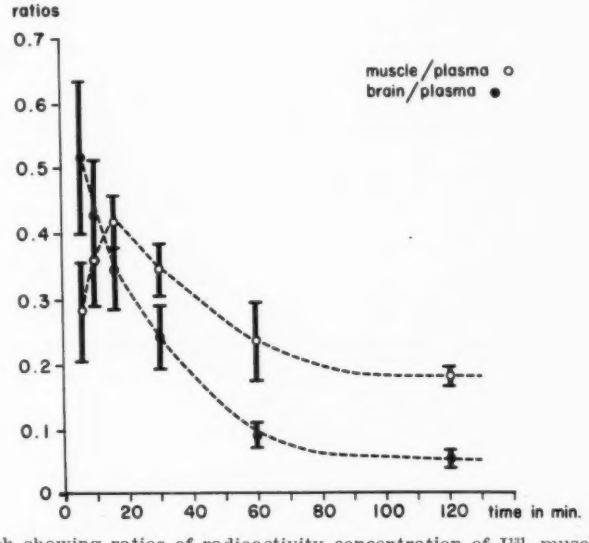

Fig. 1.—Graph showing ratios of radioactivity concentration of I^{131}, muscle to plasma, and brain to plasma, at various time intervals following intraperitoneal injection of radioiodinated-4-iodoantipyrine in rats. Standard deviations are shown.

RESULTS

Experiment I.—The apparent relative concentrations of 4-I^{131} AP in rat plasma, brain, and muscle are given in Table I. These data are shown graphically in Fig. 1.

Experiment II.—The relative diffusibilities of I^{131} from 4-I^{131} AP in the experimental circumstances described are shown in Table II. These data are shown graphically in Fig. 2.

In general, the results in animals pretreated with Lugol's solution and in those which received additional carrier antipyrine did not differ significantly

from the results shown in Tables I and II. These data for plasma dialysis are given in Table III.

TABLE II. RELATIVE DIFFUSIBILITY OF RADIOACTIVITY FROM 4-I^{131} AP ACROSS A CELLOPHANE MEMBRANE (STANDARD DEVIATIONS ARE GIVEN)

GROUP	NO. OF EXPERIMENTS	PER CENT RADIOACTIVITY REMAINING IN MEMBRANE
A, I^{131} AP plus plasma in vitro	6	50.3 ± 3.1
B, I^{131} AP plus H_2O	6	30.2 ± 2.9
C, I^{131} AP in plasma in vivo, 15 minutes	6	16.8 ± 2.6
D, Same as above, 1 hour	6	6.7 ± 2.9
E, Same as above, 2 hours	6	4.2 ± 1.2

TABLE III. RELATIVE DIFFUSIBILITY OF RADIOACTIVITY FROM PLASMA ACROSS CELLOPHANE MEMBRANE IN ANIMALS PRETREATED WITH LUGOL'S SOLUTION, AND IN THOSE RECEIVING ADDED ANTIPYRINE. *t* TEST COMPARES THESE DATA WITH DATA IN COLUMN ONE (TAKEN FROM TABLE II. (STANDARD DEVIATIONS ARE GIVEN)

DATA FROM TABLE II		LUGOL'S PRETREATED			ADDED ANTIPYRINE		
NO. RATS	PER CENT UNDIFFUSED	NO. RATS	PER CENT UNDIFFUSED	P	NO. RATS	PER CENT UNDIFFUSED	P
8 (1 hour)	6.7 ± 2.9	6	7.4 ± 3.8*	< .8	6	5.4 ± 1.5	< .4
8 (2 hours)	4.3 ± 1.2	6	6.0 ± 1.0	< .02	6	4.5 ± 1.1	< .7

*Large S. D. due to one experiment showing 14 per cent undiffused radioactivity.

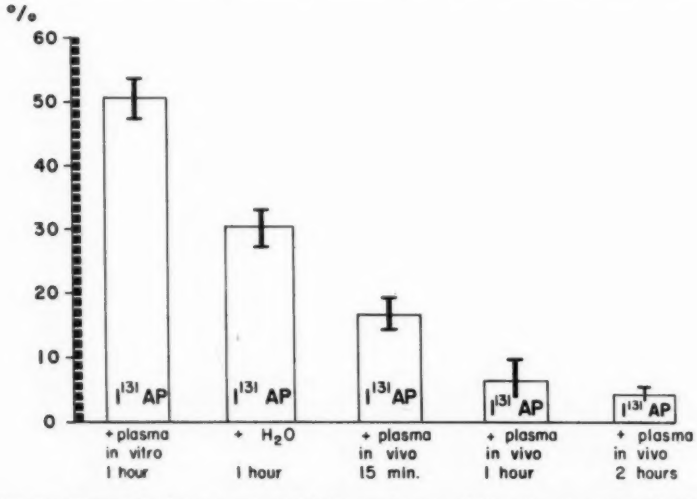

Fig. 2.—Graph of results (per cent of total counts remaining in membrane) of 1 hour dialysis of various mixtures of radioiodinated-4-iodoantipyrine. Standard deviations are shown.

DISCUSSION

Measurements of tissue radioactivity made at 30, 60, and 120 minutes after administration of 4-I^{131} AP indicated a significantly higher concentration of 4-I^{131} AP in rat muscle than in rat brain. This substance is supposed to distribute itself in the total body water. Therefore, after equilibrium has been reached, certainly by 2 hours, it should be found in each tissue in proportion

to the water content of that tissue. Since rat muscle contains about 76 per cent water and rat brain approximately 80 per cent,[5] one would expect a slightly higher concentration of the tracer to be found in rat brain. Our observations indicated the opposite, leading to the possibility of a "blood-brain barrier" existing for 4-I^{131} AP.

To test this possibility, studies were performed to determine the relative distribution of the radioactivity from 4-I^{131} AP at shorter intervals following administration. These studies indicated that during the first 10 minutes after administration a higher concentration of the tracer was found in brain than in muscle. The concentration in brain probably rose more sharply because of its greater blood flow. Determinations made 5 minutes after injection indicated that the concentration in the rat brain had already reached its maximum, and was now decreasing rapidly. Approximately equal concentrations were found in the brain and muscle between 10 and 15 minutes following injection, after which time the concentration of the tracer in muscle also began to fall. Brain concentration continued to fall, but at a much faster rate than did muscle concentration. The first 30 minutes or so of this phenomenon can be explained by the higher cerebral blood flow and the falling plasma concentration. At 1 hour, and certainly at 2 hours, the behavior of 4-I^{131} AP is not that of a substance distributed in total body water.

The possibility that all of the I^{131} tag might not remain attached to the antipyrine molecule now had to be considered. If this were present as free iodine, or as part of another compound to which a blood-brain barrier exists, it would explain the results of Experiment I. As the 4-I^{131} AP left the brain and was metabolized, little or none of the new I^{131}-containing compound would pass the blood-brain barrier on recirculation, but would diffuse into muscle, accounting for the high concentration of radioactivity found there.

The results of Experiment II support this suggestion. The results in Group B indicate the rate of diffusion for the 4-I^{131} AP molecule alone. The slower rate observed in Group A demonstrates the known fact that 4-I^{131} AP, like antipyrine, is bound (reversibly) to plasma protein. The results in Groups C, D, and E indicate that following administration of 4-I^{131} AP, increasing amounts of a more diffusible form of I^{131} are found in the plasma. Blocking the thyroid uptake of I^{131} does not significantly alter these observations, nor does the use of additional carrier antipyrine.

These data suggest that within a few minutes after injection of 4-I^{131} AP, significant and increasing quantities of the I^{131} tracer are detached from the antipyrine molecule in a form which is more diffusible than 4-I^{131} AP. Yet this new form does not pass freely the blood-brain barrier.

The metabolism of antipyrine in man[6] suggests the possible fate of 4-I^{131} AP in the rat. Following administration of antipyrine to man, 30 per cent of the administered dose is hydroxylated at the 4′ position to form hydroxyantipyrine, which is immediately conjugated with glucuronic or sulfuric acid and excreted in the feces. Since the I^{131} tag is placed at the 4′ position of antipyrine, it is possible that hydroxylation at this position releases I^{131}, which then either joins the iodine pool or is incorporated into a new smaller molecule.

Hansen[7] has found that in cats as well, I^{131} is rapidly released from 4-I^{131} AP. Further, he has noted that removal of the liver in cats prevents this phenomenon. It might be assumed, therefore, that the hydroxylation reaction described above occurs in the liver.

It is possible that this phenomenon occurs in man. In the figure published by Talso and associates,[2] the slope of decay of 4-I^{131} AP in man is steeper than that of antipyrine, despite the fact that these substances do not measure significantly different volumes of distribution.

At the present time it is reasonable to conclude that there are qualitative differences in the behavior of antipyrine and 4-I^{131} AP when injected in experimental animals, and that these differences as noted in the rat can invalidate the use of 4-I^{131} AP as a substitute for the lipoid soluble and widely diffusible antipyrine.

These results do not bear on the validity of the 4-I^{131} AP technique for the measurement of cerebral blood flow in the rat. This method, described by Sapirstein and Hanusek,[4] is based on the fact that 4-I^{131} AP enters the brain in considerable quantity within a few seconds of its administration. The measurements are accomplished within the minute following administration of 4-I^{131} AP and thus prior to any alteration in its structure.

SUMMARY

When injected intraperitoneally into the rat, radioiodinated 4-antipyrine loses a significant part of its I^{131} tag after 30 minutes. The I^{131} is then present as iodide or as part of a smaller molecule, which dialyzes more rapidly than 4-iodoantipyrine. This new compound does not distribute itself in the total body water, and does not freely cross the blood-brain barrier while diffusing readily into muscle, which qualities account for the much greater concentration of I^{131} found in muscle than in brain at 2 hours after intraperitoneal injection of 4-I^{131} AP into rats.

The authors are grateful to Dr. Louis Sokoloff, National Institute of Mental Health, for his helpful criticism, and to Ralph Coan and Sandra Butler for their valuable technical assistance.

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LABORATORY METHODS

A RAPID QUANTITATIVE PAPER ELECTROPHORETIC TECHNIQUE FOR DETERMINING A_2 HEMOGLOBIN

THELMA JOHNSON, B.S., AND O'NEILL BARRETT, JR., CAPTAIN, MC, USA
WASHINGTON, D. C.

A simplified paper electrophoretic technique for the determination of A_2 hemoglobin is described. It is simple, rapid, and reliable, and can be performed with equipment routinely utilized for hemoglobin and serum protein electrophoresis. A characteristic pattern is usually seen in cases of thalassemia trait, which allows for a visual prediction of increased amounts of A_2 hemoglobin, and quantitative estimates can be made with the use of a standard densitometer. The technique correlates well with results obtained with the starch block technique.

SEVERAL investigators[1-3] have demonstrated an increased amount of A_2 hemoglobin in patients with thalassemia trait and have indicated the diagnostic value of such levels in this condition. A number of techniques for the determination of A_2 hemoglobin have been described, utilizing both starch techniques[1, 2, 4] and paper electrophoresis.[5, 6] While quite reliable, these techniques either consume a good deal of time or require special preparation. We wish to describe a paper electrophoretic technique for the determination of A_2 hemoglobin which is simple, rapid, and reliable. It can be adapted by any laboratory capable of performing routine hemoglobin and serum protein electrophoresis.

MATERIALS AND METHODS

Hemolysates are prepared as 50 per cent glycerol solutions[7] with an approximate concentration of 7 Gm. per cent. Three-thousandths of a milliliter is then placed on a 30 by 3 cm. filter paper strip in a Durrum-type electrophoretic cell. Electrophoresis is performed for 3 hours, using a barbital buffer pH 9.1 with ionic strength 0.037, at 350 V. A separation of 3 cm. is obtained. Strips are then dried and stained with bromphenol blue for 15 minutes. Quantitation of the sample is performed with a standard densitometer and automatic integrator.* In the absence of a densitometer, visual scanning of the strip will usually allow for a prediction of increased amounts of A_2 hemoglobin.

Electrophoresis was performed on hemolysates of sixty-seven normal individuals and eighteen subjects with thalassemia trait. Starch block electrophoresis was performed on all samples for comparison with the paper technique.[1]

From the Department of Hematology, Division of Medicine, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.

Received for publication Sept. 30, 1960.

*Spinco Model R.: Paper Electrophoresis System Institution Manual, RIM—4.

RESULTS AND DISCUSSION

Examination of the unstained strip reveals a single, homogenous band. Staining with bromphenol blue reveals 3 areas of concentration of dye (Fig. 1). Each of these areas shows a positive reaction when stained with benzidine. The dense, homogenous band at the point of maximum migration corresponds to the A_1 hemoglobin fraction. Just ahead of the application site is a faint but definite band, visible on most strips, which we feel may correspond to the A_4 pattern described by Masri and associates.[2] The positive reaction with benzidine suggests that it is not the nonhemoglobin protein x of Derrien.[8] This area is expressed as per cent A_4 hemoglobin on the figures, but should probably be considered as that per cent of the total hemoglobin content not migrating as A_1 or A_2. It likely contains not only A_4 hemoglobin but some of the faster-moving components which have been absorbed by the paper. Midway between these two bands is another area of increased density, representing the A_2 fraction.

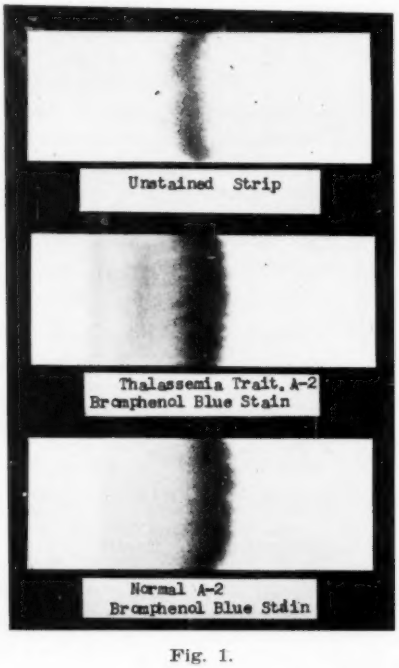

Fig. 1.

In normal specimens there is usually only a diffuse distribution of the stain, although at times a faint band can be detected (Fig. 1). In thalassemia trait, on the other hand, a distinct and easily identifiable band is discernible in most cases and allows for a visual prediction of an increased amount of A_2 hemoglobin (Fig. 1). Fresh hemolysates, not over a week old, should be used. When the strips are processed through the densitometer, both a qualitative and quantitative difference is noted. In thalassemia trait, the densitometer tracing shows a single, sharp peak in the A_2 area (Fig. 2) which is not seen in normal patterns (Fig. 3). Quantitative determination of the A_2 content reveals a significant difference between normal patterns and those of thalassemia. In normal

subjects the mean A_2 value with 2 standard deviations was 11.00 ± 3.9 per cent, while in thalassemia it was 19.82 ± 5.7 per cent. This difference is highly significant ($p = < 0.001$). All cases showing increased levels of A_2 on paper also had equivalent increased values by the starch block determination. In no case did the paper technique fail to detect elevated A_2 levels when such increases were demonstrated by the starch block technique.

It should be noted that there is a discrepancy between the absolute values obtained with this technique and those obtained by the starch block method. It seems probable that absorption of A_1 hemoglobin by the paper during the course of migration accounts for the increased values noted here. Despite this difference, however, there is a good correlation between the two methods ($r_{16} = 0.770$; $p = < 0.001$).

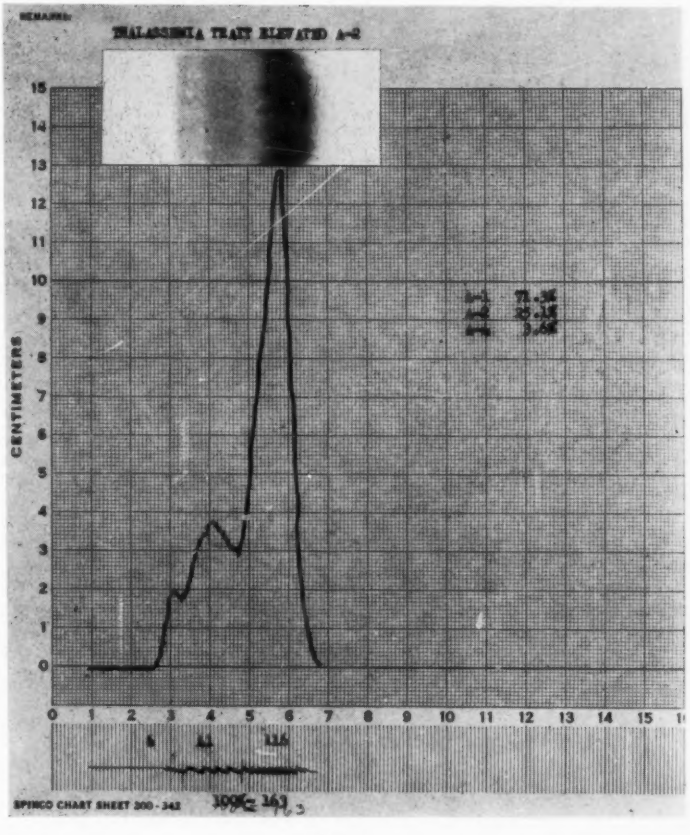

Fig. 2.

To ascertain that increased amounts of fetal hemoglobin did not account for some of the increased A_2 levels on paper, cord blood containing approximately 50 per cent F hemoglobin was run by this technique. In 3 specimens so studied, no increase in the A_2 band was noted, suggesting that even large amounts of F hemoglobin will not spuriously increase the A_2 levels by this

technique. The quantitative results of this technique are highly reproducible. In a blind study done in quadruplicate on 6 hemolysates, no variation greater than 0.5 per cent was found among the replicate determinations (Table I).

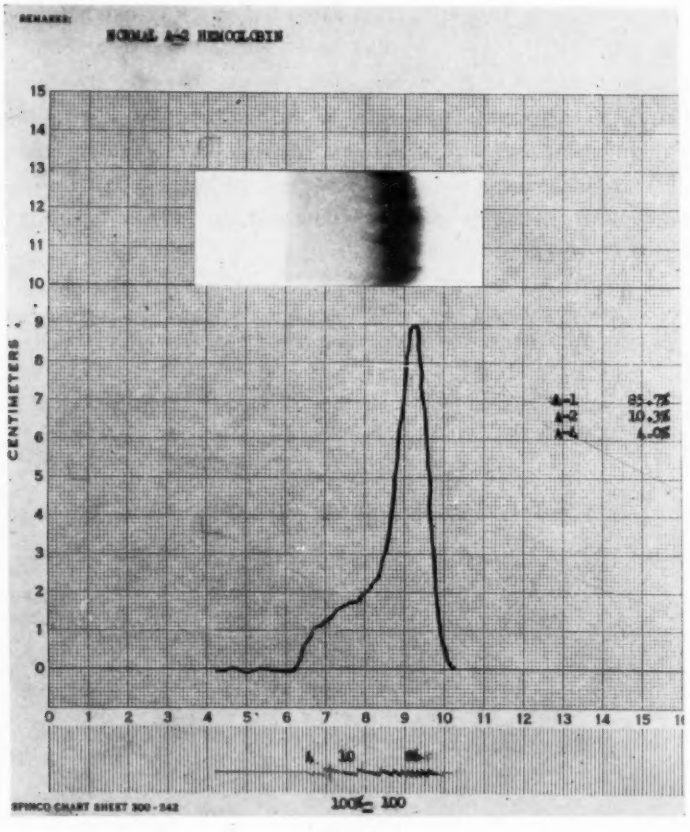

Fig. 3.

TABLE I. REPLICATE DETERMINATIONS OF A_2 HEMOGLOBIN LEVELS IN 6 SUBJECTS SHOWING REPRODUCIBILITY OF RESULTS

SUBJECT	A_2 HEMOGLOBIN DETERMINATIONS—PER CENT OF TOTAL			
	NO. 1	NO. 2	NO. 3	NO. 4
C. W.	12.1	12.5	12.0	12.0
J. G.	11.6	11.4	11.5	11.4
J. M.	12.7	12.5	12.9	12.5
M. D.	13.5	13.5	13.5	13.3
L. S.	10.8	10.4	10.5	11.0
A. Y.	11.0	11.1	11.3	11.4

SUMMARY

A simple, rapid, and reliable technique using paper electrophoresis for the determination of A_2 hemoglobin has been described. It is readily applicable to any laboratory capable of performing routine hemoglobin and serum protein electrophoresis.

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AN ENZYMATIC FLUOROMETRIC METHOD FOR THE DETERMINATION OF LACTIC ACID IN SERUM

MAURICE E. LOOMIS, M.A.*
PALO ALTO, CALIF.

In studies on carbohydrate metabolism a need has arisen for a more accurate and rapid method for the determination of lactic acid than has so far been available. A simplified adaptation to fluorometry of existing spectrophotometric methods for determination of the presence of serum lactic acid is presented which is based on the conversion of L-lactate to pyruvate with the use of excess lactic acid dehydrogenase and diphosphopyridine nucleotide at pH 10.5. The reaction is carried to completion at this pH when semicarbazide is added to fix the pyruvate. The reduced diphosphopyridine is then measured by its native fluorescence.

A substantial decrease in experimental error has been achieved by a reduction of the number of steps, including the necessity for preliminary deproteinization, used in other determinations. Values obtained by this method compare favorably with those obtained from the widely used chemical determination of Barker and Summerson.

IN studies on the in vitro carbohydrate metabolism of human leukocytes there has arisen a need for a more accurate and rapid determination of lactic acid than the widely used method of Barker and Summerson.[1] Pfleiderer and Dose[2] have described an enzymatic method, dependent upon the conversion of lactate to pyruvate, which uses an excess of lactic acid dehydrogenase (LDH) and diphosphopyridine nucleotide (DPN) in a bicarbonate buffered system at pH 9.7. The increased light absorption by reduced DPN (DPNH) is read spectrophotometrically at 366 mμ. Horn and Bruns[3] have subsequently modified this method to overcome the unfavorable equilibrium by trapping the pyruvate with semicarbazide hydrochloride and raising the pH to 10.5, which favors the forward reaction.

The proposed method here adapted for clinical determination of serum lactic acid simplifies the procedure and increases the sensitivity and range of the above methods. By measurement of the native fluorescence of DPNH with the method described by Lowry and associates,[4] the need for deproteinization of the serum has been eliminated.

From the Palo Alto Medical Research Foundation, Palo Alto, Calif.

Supported in part by United States Public Health Service Grant E-1480 and in part by a grant made to Dr. Cutting B. Favour by the California Tuberculosis and Health Association.

Received for publication June 20, 1960.

*Present address: Department of Medical Microbiology, Stanford University, Stanford, Calif.

MATERIALS AND METHODS

Reagents.—

1. Lithium DL-*Lactate.*—Distilled water, 2.13 Gm. per liter (equivalent to 100 mg. per 100 ml. L-lactic acid) is used as the stock lactic acid solution in the preparation of standards.

*2. Lactic Acid Dehydrogenase (LDH).**—Type I LDH crystallized from rabbit muscle (60,000 units per milligram protein) is used.

*3. β-Diphosphopyridine Nucleotide.**—DPN is obtained from yeast (98 per cent).

4. Glycine Buffer.—Use 0.1 M glycine containing 5 Gm. semicarbazide per 100 ml. adjusted to pH 10.5 with NaOH.

5. Buffer-Enzyme Mixture.—Shortly before the determination is to be undertaken, add 0.5 mg. LDH and 2.0 mg. DPN per milliliter of glycine buffer. One milliliter of this enzyme-buffer mixture is needed for each test serum and each standard.

6. *Diluent.*—Use 0.02 M NaOH containing 0.35 Gm. ethylenediaminetetraacetic acid disodium salt (EDTA) per liter. EDTA is used to bind heavy metals which tend to produce an inconsistent enhancement in the native fluorescence of DPNH.[4] This solution also serves to stop the reaction at the end of the incubation period.

Procedure.—

1. Make up standard solutions in the range of 0 to 50 mg. per 100 ml. lactic acid using the stock solution. Use distilled water for the blank.

2. Place 0.1 ml. (use 0.1 or 0.2 ml. pipette) of each serum† and each standard solution carefully into the bottom of a test tube with a capacity of at least 20 ml. One additional tube containing 0.1 ml. of a random serum is set up to serve as a serum control.‡

3. To all tubes except the serum control, add 1 ml. of the enzyme-buffer mixture (an automatic pipette helps to minimize variations). Mix the contents by shaking. Avoid frothing of the mixture.

4. Let stand at room temperature for 90 minutes.

5. With the use of an automatic pipette dilute all tubes, including the serum control tube, uniformly with 15 to 25 ml. of diluent. The quantity of diluent to be used depends upon the sensitivity of the fluorometer§ employed. To the serum control, add 1 ml. of the enzyme-buffer mixture. Mix all tubes.

6. Adjust the sensitivity of the machine to the range of standards employed (0 to 50 mg. per 100 ml. lactic acid). Read all test sera and the serum control. Plot a standard curve on arithmetic paper. Deduct the difference between the serum control and the blank from each of the test serum readings. Determine the values for the test sera from the standard curve.

RESULTS

Table I demonstrates the high degree of reproducibility obtained in triple determinations of 6 standard solutions containing 2.5 to 50 mg. per 100 ml. L-lactic acid and triplicate determinations on 7 normal human sera. Standard lactic acid triplicates varied on the average by 1.0 per cent while sera varied by 0.8 per cent. This compares favorably with the reported reproducibility of 2 per cent for the chemical method.[1] Data on the recovery of various amounts of added L-lactic acid in fresh pooled human serum are summarized in Table II.

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*Sigma Chemical Co., St. Louis, Mo.

†Blood should be chilled immediately upon withdrawal and serum removed within an hour to prevent further glycolysis. The serum must be free of cells and hemoglobin.

‡One serum control tube is sufficient for each series of tests, since the fluorescence of unreacted sera does not vary.

§Coleman Model 12C Fluorometer with standard "B_1" filters (Coleman primary filter UV-1 and secondary filter PC-2) were found to be satisfactory. Any fluorometer with sensitivity sufficient for the particular purpose is satisfactory.

These data compare values obtained by the standard procedure of Barker and Summerson[1] with those of the proposed method. Average recovery was 100.8 per cent with the proposed method and 106.0 per cent with the chemical procedure. In our laboratory the proposed method is both more accurate and more reproducible than the chemical analysis of Barker and Summerson.[1]

TABLE I. REPRODUCIBILITY OF THE PROPOSED METHOD

SAMPLE	EXPERIMENTS 1	2	3	PER CENT DEVIATION FROM MEAN
L-lactic Acid mg./100 ml.—*				
2.5	6.7†	6.7	7.0	1.9
5	13.7	14.2	13.9	1.2
10	25.5	25.5	26.2	1.2
20	46.5	47.5	47.7	1.1
30	65.0	65.0	65.2	0.2
50	88.5	89.5	88.7	0.5
Serum number.—				
1	20.4‡	20.4	20.5	0.2
2	20.1	20.1	19.7	0.9
3	22.9	22.9	22.6	0.6
4	24.4	24.4	24.1	0.5
5	26.6	26.6	25.4	2.0
6	16.7	16.9	16.7	0.6
7	19.8	20.1	20.1	0.7

*Equivalent solutions of lithium DL-lactate (2.13 Gm. lithium DL-lactate is equivalent to 1.0 Gm. L-lactic acid).

†Fluorometer scale readings.

‡For convenience in this study the sera used for determinations given in this table were stored for 5 days at 4° C. These higher than normal values demonstrate the necessity of using fresh sera for determination of true blood lactic acid value.[1, 3] These figures represent milligrams per 100 ml. of serum.

TABLE II. COMPARISON OF RECOVERIES OF ADDED LACTIC ACID TO HUMAN SERUM

ALIQUOTS OF POOLED SERUM	MG. PER CENT L-LACTIC ACID ADDED	BARKER AND SUMMERSON CHEMICAL METHOD (MG. PER CENT)	PROPOSED METHOD (MG. PER CENT)	PER CENT RECOVERY BARKER AND SUMMERSON	PER CENT RECOVERY PROPOSED METHOD
1	0	9.9*	9.5†		
2	2.5	13.7	11.8	132	92
3	5	14.8	14.5	98	100
4	10	18.4	20.0	85	105
5	15	23.6	25.0	91	103
6	20	31.5	30.0	108	103
7	25	35.4	35.0	102	102

*Average of duplicate determinations.

†Single determination.

DISCUSSION

In the procedure it was suggested that the volume of diluent added be in the range of 15 to 25 ml. When this amount is used, fluorescence of samples containing 0 to 30 mg. per 100 ml. L-lactic acid is directly proportional to concentration. At higher concentrations[4] readings are no longer proportional because of absorption of the exciting light within the sample. Velick[5] has shown that a shift in the absorption spectrum occurs when DPNH dissociates from the enzyme complex. This would tend to alter the linear relationship as more

DPNH is formed. Because of these inherent characteristics of the fluorometric measurement of DPNH the plotting of a curve from standards is advisable. Uniformity of pH and temperature is necessary, since these two factors influence the reaction rate and the fluorescence of DPNH.

Meister[6] found that muscle LDH was not entirely specific in that certain α-keto and α, γ-diketo acids are reduced at a pH near neutrality. LDH, however, attacks these acids at substantially lower rates than pyruvic acid. This fact, together with the relatively low concentration of the hydroxy analogues of these substances in blood, and their different pH optima, would indicate that, under the conditions proposed, their contribution to the formation of DPNH is insignificant.

Evidence to support this assumption is presented in the comparison of serum lactate values for the proposed method with those of Barker and Summerson,[1] in which the latter values were slightly higher. Bonting[7] in an extensive evaluation of the Barker and Summerson method, has shown that a number of interfering substances, notably pyruvate, are not removed and may give falsely high results.

SUMMARY

A simplified, highly reproducible and sensitive enzymatic method for the quantitative determination of serum lactic acid is presented. This method is based on the essentially complete conversion of L-lactate to pyruvate, which is fixed by semicarbazide, with the use of excess lactic acid dehydrogenase (LDH) and diphosphopyridine nucleotide (DPN) at pH 10.5. The formation of reduced DPN is measured by its native fluorescence. The data presented provide evidence for a favorable comparison of this enzymatic method with the widely used chemical determination of Barker and Summerson.[1]

The author wishes to express his appreciation to Dr. Cutting B. Favour for his support, cooperation, and advice, and to Robert W. Shirley, for his technical assistance.

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AN AUTOMATED DETERMINATION OF GLUCOSE UTILIZING A GLUCOSE OXIDASE–PEROXIDASE SYSTEM

JOHN B. HILL, M.D., PH.D., AND GERALD KESSLER, PH.D.
CHAPEL HILL, N. C., AND CHAUNCEY, N. Y.

A completely automated glucose oxidase–peroxidase procedure for the determination of glucose is described. Factors influencing the reactions utilized in this procedure have been evaluated. Results of analyses with the automated enzyme method, a manual enzyme method, and an automated alkaline ferricyanide reducing procedure are presented.

A PARTIALLY automated system utilizing glucose oxidase for the determination of glucose has previously been described.[1] Filtrates of samples and standards were manually prepared and the rate of change of absorbancy of each aliquot fed into the system was automatically determined. In this report, complete automation of a glucose oxidase–peroxidase system is accomplished by the use of an instrumental system described by Skeggs,[2] the AutoAnalyzer, which performs repetitive or continuous analyses of unmeasured samples.

Glucose oxidase (notatin) was first described by Muller,[3] and its specificity for β-glucose was shown by Keilin and Hartree.[4, 5] The hydrogen peroxide resulting from the catalytic action of glucose oxidase on β-glucose was measured by Keston[6] and Teller[7] by the oxidation of *o*-dianisidine in the presence of horseradish peroxidase. The above reactions are utilized in the presently described method.

MATERIALS AND METHODS

The instrumental system utilized for the automated methods is the AutoAnalyzer manufactured by Technicon Instruments Corporation, Chauncey, N. Y.

A. Automated Glucose Oxidase–Peroxidase Method.—

In the automated enzyme procedure, as illustrated in Fig. 1, samples are diluted with an air-segmented stream of isotonic (1.61 per cent) sodium sulfate. This reagent stream is mixed and then dialyzed against an air-segmented stream of isotonic sodium sulfate containing Triton X-100. The enzyme-dye mixture is added to the dialysate, and the combined stream is mixed and then incubated at 37° C. The absorbancy of the solution is measured at 460 mμ in a flow cuvette with a 10 mm. light path.

Reagents.—

1. Sodium sulfate: Anhydrous Na_2SO_4, 16.1 Gm. per liter is used.

2. Sodium sulfate plus Triton X-100: Anhydrous Na_2SO_4, 16.1 Gm., and 5 ml. Triton X-100* per liter are used.

From The Department of Pharmacology, University of North Carolina, Chapel Hill, N. C., and Research Laboratories, Technicon Instruments Corporation, Chauncey, N. Y.

Supported in part by Research Grant No. (A-1026) from The National Institute of Arthritis and Metabolic Diseases, The National Institutes of Health, United States Public Health Service.

Received for publication Nov. 22, 1960.

*Iso-octyl phenyl ether of polyethylene glycol, Rohm & Haas Company, Philadelphia, Pa.

3. Enzyme preparation: Twenty-five grams (1,500 u. per gram) glucose oxidase* and 200 mg. horse-radish peroxidase† are well mixed with 1 L. 0.1 M phosphate buffer of pH 7.0. This suspension is then filtered through Whatman No. 1 paper on a Buchner funnel. A clear, straw-colored enzyme solution results, pH 7.0 ± 0.05. This solution is stored in an amber bottle and kept refrigerated when not in use. It is brought to room temperature immediately before use.

4. Dye: Five hundred milligrams *o*-dianisidine‡ is dissolved in 100 ml. ethanol. Two hundred fifty milliliters of glycerol is then added and the mixture diluted to 1 L. with distilled water. The solution is filtered and stored in an amber bottle. If this solution becomes turbid, it is refiltered before use.

5. Glucose standards: Standards of 25, 50, 100, 150, 200, 250, and 300 mg. per 100 ml. of saturated benzoic acid are usable for at least 6 months when kept at room temperature. No differences in absorbancy were noted when standards prepared in a solution of saturated benzoic acid were compared with standards in water.

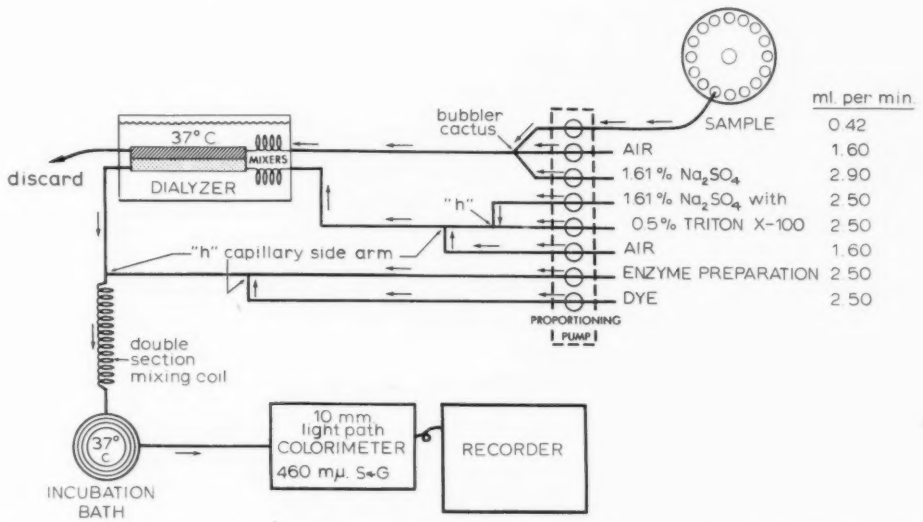

Fig. 1.—Flow diagram for determination of glucose by the glucose oxidase-peroxidase method.

Calculations.—Glucose concentrations are calculated from the average of 2 sets of standards, one of which is run immediately before and one immediately after the specimens to be analyzed. The resulting 2 sets of points are plotted on graph paper, and a calibration curve is constructed by connection of the average of the points at each concentration of glucose standard. This minimized any error owing to deviations in the position of the standard curve. These deviations have ranged from 1 to 6 mg. per 100 ml., with an average of 4 mg. per 100 ml. The deviation has always been in the direction of an increase in absorbancy.

B. Manual Glucose Oxidase–Peroxidase Method.—

The manual glucose oxidase procedure employed is a microtechnique. To 1.0 ml. distilled water is added 0.02 ml. of sample, 0.25 ml. $Ba(OH)_2$, and 0.25 ml. $ZnSO_4$. The mixture is shaken and centrifuged. One milliliter of the supernatant solution is added to 2.0 ml. of an enzyme-dye solution, and the mixture is allowed to stand at room temperature for

*DeeO, Miles Chemical Company, Clifton, N. J.

†Two thousand units per microgram, or RZ unit of 1 or more, Worthington Biochemical Corporation, Freehold, N. J.

‡3, 3′ Dimethoxybenzidine, Eastman Organic Chemicals, Rochester, N. Y.

50 minutes, at which time one drop of 4 N HCl is added to stop the reaction. The absorbancy of the solution is read in a Beckman Model B spectrophotometer at 395 mμ in a cuvette with a 10 mm. light path. Samples, standards, and reagent blanks are run simultaneously.

Reagents.—

1. Barium hydroxide: Ten grams $Ba(OH)_2 \cdot 8\ H_2O$ per liter is used.

2. Zinc sulfate: Ten grams $ZnSO_4 \cdot 7\ H_2O$ per liter is used. The concentration is adjusted as described by Somogyi.[8]

3. Enzyme-dye solution: Five milligrams *o*-dianisidine is dissolved in 5 ml. ethanol and then added to 95 ml. 0.05 M phosphate buffer, pH 7.0, containing 5 mg. peroxidase and 125 mg. glucose oxidase. The solution is filtered and kept refrigerated when not in use.

4. Hydrochloric acid: A 4 N solution of this acid is used.

5. Glucose standards: 20 and 40 μg glucose per 100 ml. water are freshly prepared from a stock standard containing 1 mg. glucose per milliliter water saturated with chloroform.

C. Automated Alkaline Ferricyanide Reducing Method.—

The chemical procedure for glucose measures total reducing substances and employs alkaline potassium ferricyanide and potassium cyanide as recommended by the Technicon Instrument Corporation. The automated methodology is a modification of a manual procedure by Hoffman,[9] and has been described by Johnson[10] and Grady and Lamar.[11] The flow diagram of the method used in this study has not been previously published. Fig. 2 gives the manifold and reagent system.

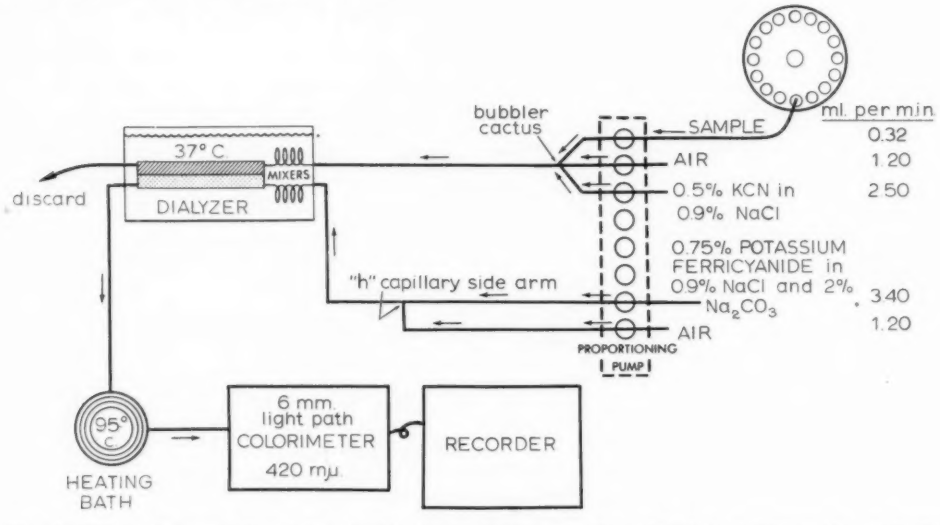

Fig. 2.—Flow diagram for determination of glucose by the alkaline ferricyanide reducing method.

RESULTS

Factors Which Influence the Automated Enzymatic Procedure.—

1. pH.—The investigation of Keilin and Hartree[4, 5] showed the pH optima for a glucose oxidase–catalase system to be 5.6. Our preliminary experiments utilizing a glucose oxidase–peroxidase coupled enzyme system were in agreement. Commercial preparations of glucose oxidase were found to have amylase and maltase activity at pH 5.0 to 6.9. The amount of activity of these preparations was checked with a solution of 200 mg. maltose per 100 ml. saturated benzoic

acid solution and with a purified corn syrup preparation of the same concentration. Amylase and maltase activity for these preparations was found to decrease with increasing pH; at pH 7.0 no activity was detectable. A pH of 7.0 was therefore chosen for the method, as it is being used industrially to determine glucose in the presence of maltose and other polysaccharides.

2. Enzyme Concentration.—The manifold as outlined in Fig. 1 gives an approximate reaction volume of 10 ml. per minute. For this reaction mixture 6.25 mg. glucose oxidase and 50 μg peroxidase per milliliter reaction mixture give good activity for a range of 0 to 400 mg. glucose per 100 ml., and increasing these concentrations does not change this activity appreciably.

TABLE I. REPRODUCIBILITY OF AUTOMATED GLUCOSE OXIDASE METHOD ON POOLED SERA

DAY	SERUM POOL	MG./100 ML. GLUCOSE
6/14	A	89, 90, 90, 92
6/16	A	90, 90, 91, 93
6/17	A	91, 91, 92, 92
6/20	A	89, 89, 90, 91
7/ 5	B	94, 95, 95, 95
7/ 6	B	94, 94, 95, 95
7/ 8	B	91, 93, 93, 94
7/10	B	96, 98, 98, 101
7/11	B	95, 97, 97, 98
7/12	B	96, 96, 96, 97

3. Dye.—The two dyes investigated were *o*-dianisidine and *o*-tolidine. Color developed with *o*-tolidine reached a maximum, then faded; with *o*-dianisidine, the developed color was relatively stable. Concentrations of *o*-dianisidine from 25 to 125 μg per milliliter reaction mixture give similar results. A level of 125 μg per milliliter reaction mixture was arbitrarily chosen. Both ethanol and methanol may be utilized for dissolving the dye. The concentration of alcohol as used in the dye reagent did not alter enzyme activity. A recent disclosure by Washko[12] suggests the incorporation of glycerol into the enzyme-dye mixture. Best results, in terms of freedom from contamination from one specimen to another, sensitivity, and stability, were obtained by the inclusion of glycerol in the dye solution at a concentration of 250 ml. glycerol per liter of reagent.

4. Filters.—The absorption spectrum of the reaction mixture was obtained by means of a Beckman DK recording spectrophotometer immediately after collection. The spectrum of the oxidized dye of a glucose-containing reaction mixture, with or without the addition of glycerol, gives a maximum absorbancy at 460 mμ when read against the reagent blank. The addition of 2 drops concentrated sulfuric acid to 3 ml. of blank and glucose-containing reaction mixtures shifts the peak to 400 mμ. The further addition of acid, as suggested by Washko,[12] changes the peak to 540 mμ when read against the appropriate reagent blank. At any of these maxima the absorbancy is the same when changes in volume are taken into consideration. Since there is no advantage to adding acid, it is omitted from the method, and 460 mμ filters are used.

5. Wetting Agent.—A series of wetting agents were evaluated for their ability to prevent the dye from precipitating out of the reaction mixture and

adsorbing to the tubing. Incorporating Triton X-100 at a concentration of 0.5 per cent (volume per volume) in 1.61 per cent Na_2SO_4 proved optimal.

6. Time.—As can be seen from Fig. 1, the volume of the reaction mixture is 10 ml. per minute. This flow gives an incubation time of approximately 3.5 minutes. The lag time of the system, i.e., time from aspiration of sample to appearance on the record, is approximately 7 minutes. The recommended sampling time is 40 determinations per hour. At this sampling rate the absorbancy is similar to the absorbancy obtained with continuous sampling. This means a large enough sample has been aspirated and enough time allowed for the system to reach equilibrium.

Application of the Method to the Determination of Glucose.—

1. Specificity.—Keilin and Hartree[4, 5] examined the specificity of their enzyme system at pH 5.6. A study of the specificity of the present reagent system at pH 7.0 showed no measurable "glucose" activity for the following sugars: fructose, galactose, lactose, maltose, mannose, raffinose, ribose, sucrose, xylose, and corn syrups containing glucose plus varying concentrations of polysaccharides.

TABLE II. RECOVERY OF GLUCOSE ADDED TO SERUM MANUALLY

GLUCOSE ADDED (MG./100 ML.)	GLUCOSE CALCULATED* (MG./100 ML.)	GLUCOSE DETERMINED (MG./100 ML.)	
		JULY 14, 1960	JULY 15, 1960
0		93, 95	89, 90, 91
50	141	144, 145	139, 142, 143
100	190	191, 193	184, 189, 191
150	238	234, 238	241, 244, 247

*Calculation made using the average of the 5 determinations on serum without added glucose as the endogenous glucose concentration.

2. Specimens.—Biologic specimens consisting of whole blood, plasma, serum, and cerebrospinal fluid were found to be suitable for glucose analysis by the automated enzymatic and reducing procedures as well as the manual glucose oxidase–peroxidase system. Whole blood and plasma specimens contained a potassium oxalate–sodium fluoride mixture to act as an anticoagulant and prevent glycolysis. Fluoride did not inhibit at concentrations as high as 10 mg. per milliliter. Anticoagulants such as oxylate, heparin, citrate, and Sequestrene were also satisfactory for glucose analysis. Undiluted urine samples were unsatisfactory as they inhibited the glucose oxidase–peroxidase system, as well as varied in the amount of nonglucose reducing substances which can be measured by the alkaline ferricyanide method.

3. Reproducibility.—The glucose content of the 2 pools of serum were determined on several days. The results in Table I show the method is reproducible.

4. Recoveries.—Two types of recovery experiments were performed: A. The classical approach, adding different concentrations of a substance to pooled serum, illustrated in Table II. B. The introduction of a fixed concentration of material into the diluent as outlined by Skeggs[2] described below.

1. A set of standards and unknown specimens were run by the automated glucose oxidase–peroxidase procedure as outlined in Fig. 1. The glucose contents of the samples were determined from the resultant standard curve.

2. The same standards and specimens were rerun as above with the exception that the samples were diluted with 1.61 per cent Na_2SO_4 containing a fixed concentration of glucose. The glucose contents of these rerun specimens were determined from the rerun standard curve.

The glucose concentration of a specimen run by the 2 described techniques, B 1 and B 2 above, was identical as long as the specimen contained no constituent which inhibited or accelerated the reaction. (An inhibitor was observed to give a lower result when glucose was added to the diluent.)

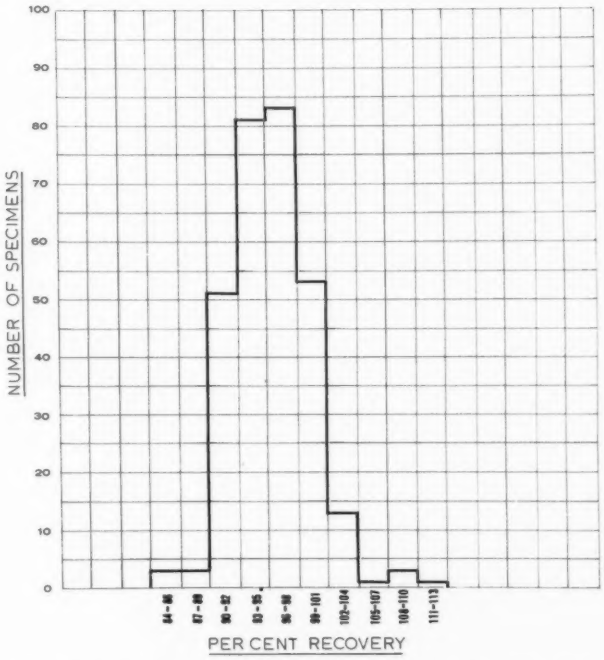

Fig. 3.—The distribution of per cent recovery of glucose for 292 clinical specimens.

To estimate the extent of inhibition the following was done: The concentration of glucose added to the specimens was determined by noting the base line (reagent blank) when glucose was added to the diluent and determining where this point falls on the standard curve obtained without glucose in the diluent. This increased amount of absorbancy is expressed in terms of milligrams per 100 ml. glucose and is called the "glucose increment." The per cent loss for a given specimen was determined in the following manner: The result (milligrams of glucose per 100 ml.) for a given specimen run without glucose in the diluent minus the result (milligrams of glucose per 100 ml.) run with glucose in diluent, divided by the "glucose increment," and multiplied by 100 gives the per cent glucose lost. A typical experiment is shown in Table III. The distribution of

the per cent recoveries run on 292 clinical specimens consisting of whole blood, plasma, serum, or cerebrospinal fluid may be seen in Fig. 3. The recoveries ranged from 90 to 104 per cent for 90 per cent of the specimens.

The effect of protein concentration and the possible presence of inhibitors or activators was further investigated by dilution experiments. Sera with elevated glucose levels were pooled and dilutions made with 0.9 per cent sodium chloride and 1.61 per cent sodium sulfate. Dilutions as high as 1:10 were prepared. When the absorbancies of the diluted specimens were plotted against the dilution ratio, the points fell on a straight line. This occurred whether the dilution was made with 0.9 per cent sodium chloride or 1.61 per cent sodium sulfate.

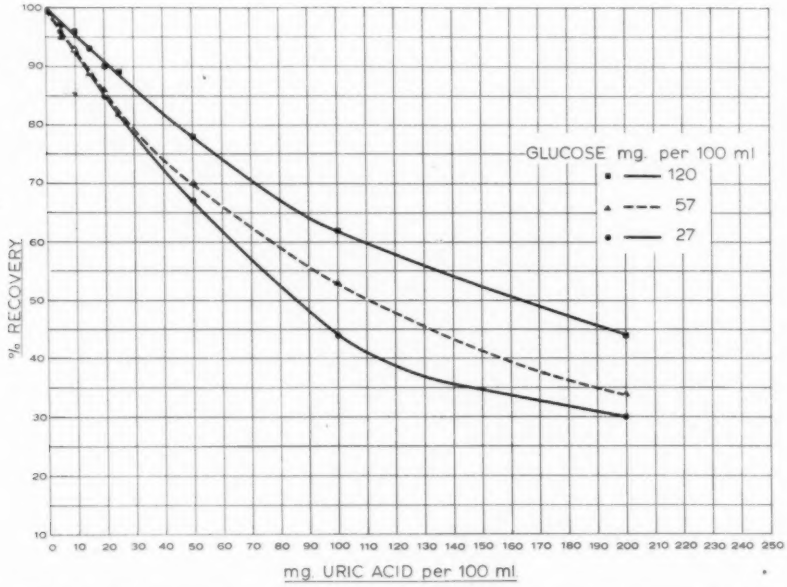

Fig. 4.—The per cent recovery of glucose, at various glucose concentrations, as influenced by the presence of varying amounts of uric acid added to the glucose solution.

TABLE III. RECOVERY* EXPERIMENT USING THE AUTOMATED ADDITION OF GLUCOSE

SPECIMEN NO.	ABSENCE OF ADDED GLUCOSE MG./100 ML.	PRESENCE OF ADDED GLUCOSE† MG./100 ML.	DIFFERENCE	PER CENT RECOVERY
134	73	71	2	95
135	67	65	2	95
136	65	65	0	100
137	71	70	1	97
138	99	97	2	95
139	99	96	3	92
140	65	62	3	92
141	143	142	1	97
142	69	67	2	95
143	73	71	2	95

*Per cent recovery $= 100 - \left[\frac{\text{Result (mg./100 ml.) without glucose in diluent} - \text{result (mg./100 ml.) with glucose diluent.}}{\text{"glucose increment"}} \times 100 \right]$

†For this experiment the "glucose increment" was 39 mg. per 100 ml.

5. Inhibition.—Inhibitors of the present system have previously been reported and were reinvestigated. The following did not inhibit the present coupled enzyme system during analysis of glucose prepared in saturated benzoic acid, or when added to plasma: sodium fluoride, 10 mg. per milliliter, bilirubin, 200 μg per milliliter, l-epinephrine bitartrate, 10 μg per milliliter, l-norepinephrine bitartrate, 10 μg per milliliter, and ascorbic acid, 25 μg per milliliter.

Uric acid, when added to the present enzyme-dye system, caused a decrease in the color developed in response to glucose. Fig. 4 shows the per cent inhibition of glucose recovery at 3 different glucose concentrations as a function of the concentration of uric acid in the sample being analyzed. As can be seen from the figure, the inhibition by uric acid which would normally be found in serum or plasma (2 to 6 mg. per 100 ml.) is of the order of 4 per cent. Results of recovery experiments for clinical samples, as in Fig. 3, show the modal loss to be approximately 4 per cent. One of the samples showing a 12.5 per cent loss on recovery was analyzed for uric acid and found to have a concentration of 18 mg. per 100 ml.

Uric acid can inhibit the recovery of glucose by 2 mechanisms. First, it is a substrate for horse-radish peroxidase, as shown by Paul.[13] That this is not the principal method of inhibition is shown by the fact that the addition of an excess of peroxidase to the system does not eliminate the inhibition. Second, uric acid appears to complex with an intermediate oxidation state of *o*-dianisidine to yield a product with a visible absorption peak at 400 mμ rather than 460 mμ. Absorbancy also is decreased per unit of glucose. This occurs whether the dye is oxidized by hydrogen peroxide in the presence of peroxidase or in a system without enzymes, such as sodium hypochlorite plus the dye. Evidence for complexing with an intermediate oxidation state of the dye is as follows:

> The oxidized dye has an absorption peak at 460 mμ. The dye oxidized in the presence of uric acid has a peak at 400 mμ. The reduced dye plus uric acid produces no visible color, and the ultra-violet absorption spectrum of such a mixture is equal to the sum of the individual ultra-violet absorption spectra of its component parts. If uric acid is added to the system after the dye has been oxidized, the peak remains at 460 mμ.

Inhibition of the system by uric acid also occurs when *o*-tolidine is used.

6. Correlations.—Specimens, collected from routine clinical laboratories, consisted of whole blood to which oxalate and fluoride had been added, plasma containing oxalate and fluoride, and serum. For storage, whole blood specimens were refrigerated, plasma and serum frozen. Table IV shows the averages of all samples run by the automated and manual glucose oxidase–peroxidase procedures and automated reducing method. Samples for the manual glucose oxidase–peroxidase determinations were chosen at random and duplicate determinations run. Any duplicate determinations not agreeing within 5 per cent or 5 mg. per 100 ml. were reanalyzed. The automated procedures were performed on single specimens.

TABLE IV. COMPARISON OF AVERAGE VALUES (MILLIGRAMS PER 100 ML.) OBTAINED ON STORED CLINICAL SAMPLES WITH THE THREE DIFFERENT METHODS

SAMPLE	NUMBER	AUTOMATED ENZYME METHOD	MANUAL ENZYME METHOD	AUTOMATED REDUCING METHOD
Whole blood	18	78.2	80.6	90.1
Plasma	10	126.3	122.4	135.1
Serum	32	90.6	92.2	101.5
All samples	60	92.8	93.8	103.7

TABLE V. COMPARISON OF FASTING GLUCOSE VALUES ON "NORMAL SUBJECTS"

SUBJECT NO.	GLUCOSE OXIDASE–PEROXIDASE METHOD	ALKALINE FERRICYANIDE (REDUCING) METHOD
Whole blood		
1	66	68
2	79	83
3	74	78
4	72	75
5	68	70
6	90	96
7	86	90
8	70	74
9	77	79
10	87	94
11	76	79
12	75	78
Average	76.7	80.3
Plasma.—		
1	79	86
2	93	103
3	90	97
4	84	89
5	81	85
6	105	112
7	101	107
8	85	93
9	93	100
10	102	110
11	90	99
12	86	91
Average	90.8	97.7
Serum.—		
1	69	76
2	87	97
3	85	92
4	80	83
5	74	77
6	99	107
7	95	103
8	81	87
9	85	90
10	97	106
11	83	91
12	82	87
Average	84.8	91.3

In addition, fasting specimens were collected from laboratory personnel and the glucose concentration determined immediately by the automated methodologies. A needle was placed in the vein and three specimens of venous blood were drawn to obtain whole blood containing oxalate and fluoride, plasma containing

oxalate and fluoride, and serum. The glucose concentration of these specimens as determined by the automated enzymatic and reducing methods is shown in Table V. These specimens also were redetermined the following day and similar results were obtained. The lower values for sera as compared with plasma are explained by the occurrence of glycolysis before separation from the clot.

DISCUSSION

Published reports[14-16] have shown varying amounts of "non–glucose reducing substances" to be present in blood when glucose oxidase–peroxidase techniques are compared with Nelson-Somogyi or other reducing methods. In this report no attempt has been made to correlate these differences.

In the classical recovery studies glucose is added in differing amounts to a pool of serum which does not change. Replicate determinations reflect the inherent variability of the method and the success or failure of recovery is dependent upon the presence or absence of interfering substances in the specific serum pool being analyzed. In the automated recovery experiments each specimen is subjected to a recovery. This is a more demanding test of a method than the usual few recovery experiments performed; it was made practical by automation.

Attempts were made to utilize the enzyme uricase to prevent the inhibition of the glucose oxidase–peroxidase enzyme system by uric acid. The end product of uricase action, allantoin, was found not to inhibit the enzyme system. When uricase was added to the reagents, on either side of the dialyzer, color was produced by uric acid. The color appeared to be proportional to the amount of uric acid in the sample and developed even in the absence of glucose. This added color masked the inhibition by uric acid when glucose was present. The addition of larger amounts of uricase gave more color for a given concentration of uric acid. The masking action of uricase did not correct for the inhibition of uric acid at all levels of glucose concentration.

A modification of the present method is available in which uric acid inhibition is absent. A distribution of recoveries similar to that shown in Fig. 3 for 23 clinical specimens analyzed ranged from 94 to 106 per cent. The average recovery, unlike the data in Fig. 3, was 100 per cent. This modification was accomplished by the addition of uric acid to the solution to be dialyzed against the specimen. As can be seen in Fig. 4, the change in recovery with respect to uric acid in the system decreases with increasing amounts of uric acid. If enough uric acid is added, the increased inhibition afforded by uric acid present in blood becomes negligible. Fifty milligrams uric acid per 100 ml. of sample produces no inhibition of recovery with this modification. As would be expected, the sensitivity of the modification is greatly decreased. This necessitated the use of decreased volumes of flow, which decreased the efficiency of the wash between specimens and required a rate of determination no greater than 20 per hour. Since it is unlikely that inhibition greater than 15 per cent due to the presence of uric acid would ever be encountered in clinical specimens, the advantages of increased rates of determination far outweigh the error in the determination.

SUMMARY

1. A flow diagram and reagent system are presented for the automated determination of glucose by a glucose oxidase–peroxidase enzyme-dye technique.

2. The influence of pH, enzyme and dye concentrations, and incubation time of the automated methodology were investigated. Specificity, reproducibility, and recovery of the method were studied. Possible inhibitory substances were reinvestigated, with uric acid showing approximately 4 per cent inhibition at normal plasma levels.

3. Comparisons between the automated and a manual glucose oxidase–peroxidase enzyme method and an automated reducing procedure were made. Results of the automated and manual enzyme procedures were similar; the automated reducing procedure gave higher values.

We wish to express our appreciation to Doris Cowart and Constance Guimarro for their excellent technical assistance, Dr. Jacob Struck, Jr., Miles Chemical Company, for supplies of purified glucose oxidase preparations, and the personnel of the clinical chemistry laboratories of the North Carolina Memorial Hospital and the Veterans Hospital, Durham, N. C., for their aid in obtaining clinical specimens.

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Department of Medicine
Barnes and Wohl Hospitals
Washington University School of Medicine
600 S. Kingshighway Blvd., St. Louis 10, Missouri

PUBLISHED BY THE C. V. MOSBY COMPANY, 3207 WASHINGTON BLVD.
ST. LOUIS 3, U. S. A.

Published monthly. Subscriptions may begin at any time.

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*Bauer, A. C. and Redner, W. J., Jr., Obst. & Gynec. 17.325, 1961

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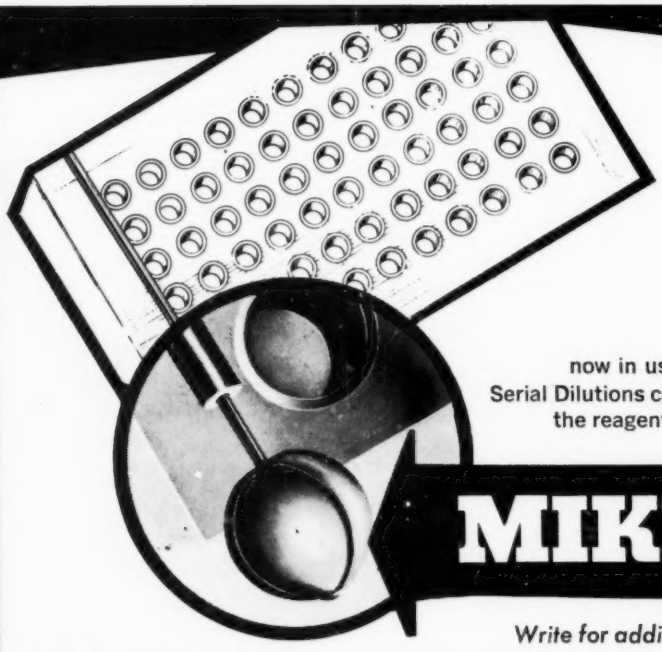

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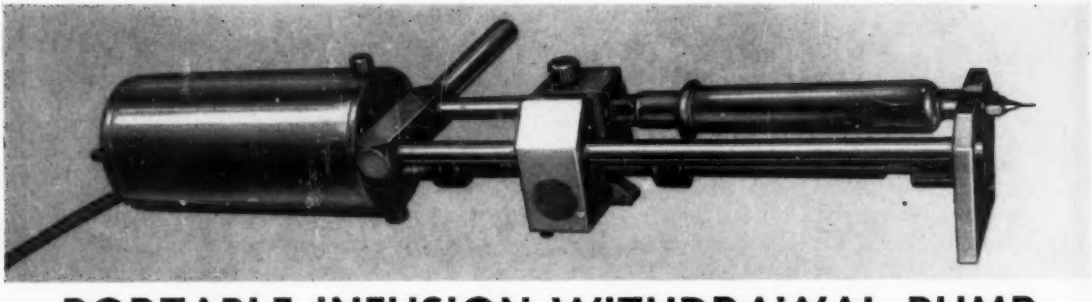

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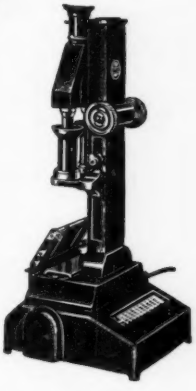

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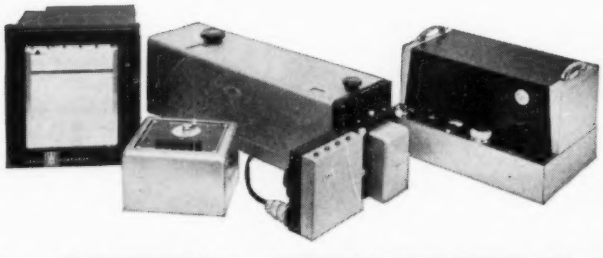

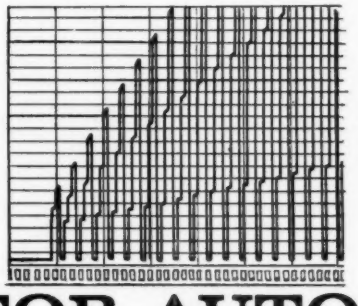

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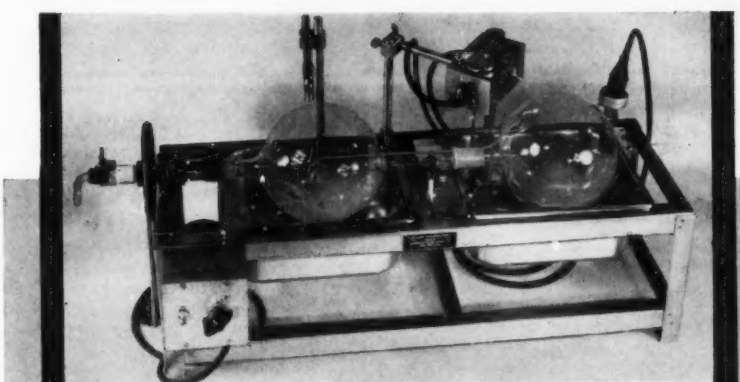

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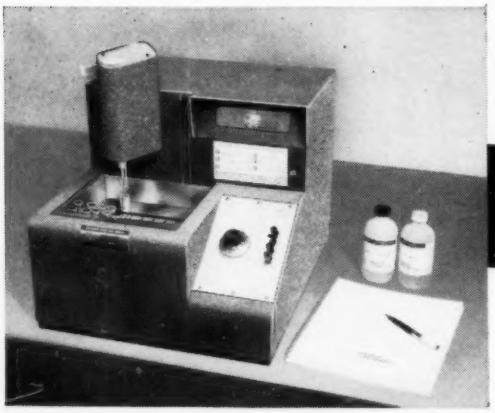

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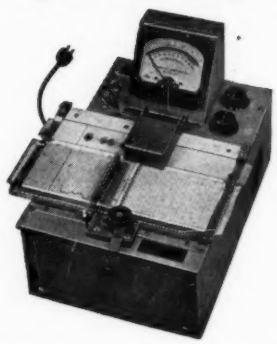

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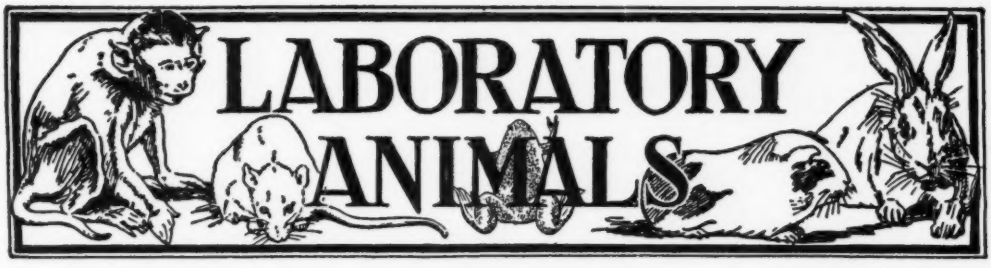

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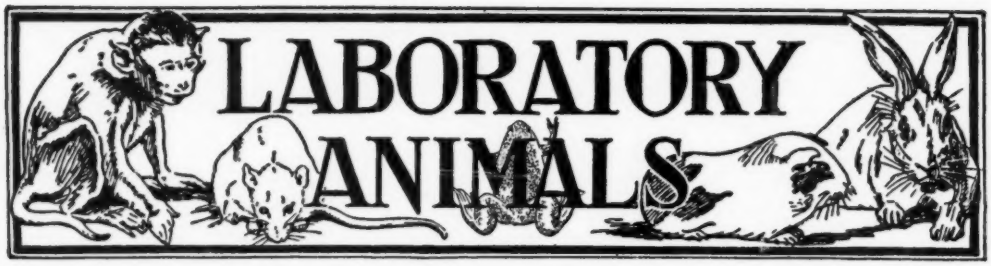

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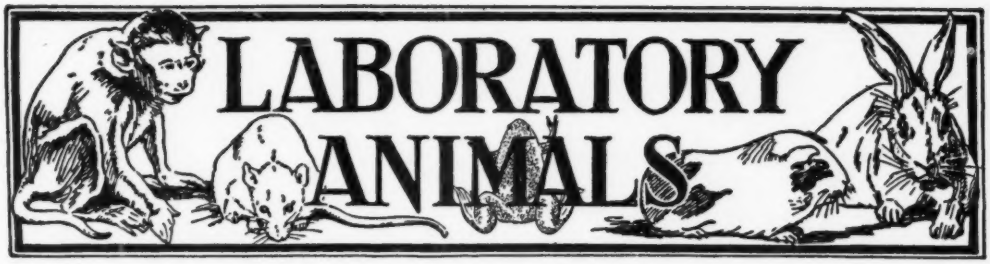

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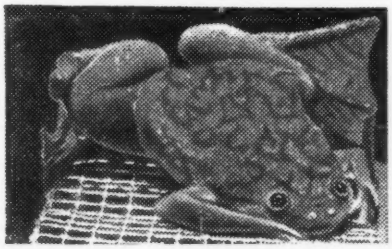

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VOLUME 57

JANUARY—JUNE, 1961

ST. LOUIS

THE C. V. MOSBY CO.

1961

981

VOLUME 57

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**Printed in the
United States of America**

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